A CONTRIBUTION TO THE DYNAMICS OF TOXICITY AND THE THEORY OF DISINFECTION

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I. INTRODUCTION

It has long been known that the effects of a toxic agent acting upon bacterial or other forms of protoplasm become manifest only after the lapse of a shorter or longer period of time. Some thirty years ago Abbott (1891), studying the disinfection of *Staphylococcus pyogenes-aureus* by mercuric chloride, concluded that the explanation for the time curve must lie with differences in the resistance of individual cells in a bacterial population to the lethal agent. Since then numerous workers have studied the course of disinfection processes by various quantitative methods with the hope that such studies might cast some light upon the nature of the reaction between toxic agent and cell and lead to an explanation of the shape of the time curve of the process. Admittedly, the physical and chemical systems involved in the structure of protoplasm are extremely complex and the reactions between a toxic agent and the constituents of these systems may be equally complex. None the less, it has been the hope of many investigators that some simple physical or chemical reaction between disinfectant and protoplasm would be found to suffice as an explanation for the dynamics of the lethal process. And some have considered that these hopes have been attained.

In recent years there have been published several reviews of the extensive literature on the theory of disinfection dynamics. Hence it is not our intention to present more than a few comments on earlier papers and only such as are especially pertinent to the purposes of the present study. Critical discussions may be

consulted in the papers of Brooks (1918–1919), Eisenberg (1918–1919), Cohen (1922), Reichenbach (1922), Falk (1923) and Fulmer and Buchanan (1923).

In their classic contribution, Krönig and Paul (1897) reported the results of careful studies on disinfection by mercuric chloride and succeeded in demonstrating conclusively that the process of disinfection is a gradual and not an instantaneous one, and that it follows an orderly sequence. From their data, Ikéda (1897) found that the curve prepared by plotting the logarithms of surviving organisms against elapsed time is a straight line and that the rate of the disinfection process is in agreement with the rate calculated from the equation of an unimolecular reaction. In a like manner, Miss Chick (1908, 1910, 1912, 1913) was able to calculate a similar, essential concordance with the logarithmic rate from the data of Clark and Gage on the disinfectant action of sunlight and from her own experiments with silver and mercury salts, phenol, heat, blood serum, etc. Numerous other workers (Madsen and Nyman, Cohen, etc.) have obtained essentially the same results. However, nearly all these workers have observed occasional deviations in the logarithmic curve from a straight line, such deviations being especially common in the earliest and latest periods of the disinfection process. Bellei, Loeb and Northrop, Eijkman, Hewlett, Reichel, Reichenbach, Brooks, Peters and others have raised objections to an interpretation of these observations in terms of a simple unimolecular reaction. Indeed, some workers have contended "that such resemblances as have been found between such curves and unimolecular reaction or logarithmic curves are superficial and fortuitous. Any method therefore of evaluating disinfecting power based upon such a concept must prove misleading."[1] With these few introductory remarks for orientation we may proceed to an examination of some new experimental data and then return to a more careful reconsideration of the theoretical aspects of the problem.

The experiments here reported were originally designed as part of an extensive study to determine the effects of certain inorganic

[1] Fulmer and Buchanan, 1923, 88.

salts upon bacterial viability and to cast some light upon the dynamics of the processes of cellular death and of disinfection. It is our plan to present for analysis here only a few of hundreds of similar experiments on the rates of dying of *Bacterium coli* in salt solutions, choosing for our present purposes the data from typical findings upon the mortality in aqueous sodium and calcium chloride solutions of various concentrations and in distilled water at various pH values.

II. EXPERIMENTAL METHODS

Our experiments were conducted with a single strain of *Bacterium coli* which was isolated from a polluted stream near New Haven in the autumn of 1916. It was strain 38 of the original collection of Winslow and Cohen (1918). This strain of *Bact. coli* maintains itself in distilled water at a favorable pH value without material decrease in numbers for a period of nearly twenty-four hours, actual increases being not uncommon during the first few hours. Occasionally, however, a particular suspension will show a marked decrease due to some cause which we have not yet determined. The details of technique used in these studies have been reported in earlier papers (Winslow and Falk, 1923a, 1923b) and need not be repeated.

III. GENERAL RESULTS

In table 1 we cite the data taken from a typical experiment, 37-C, to indicate certain characteristics of the viability curve of *Bacterium coli* in a comparatively dilute solution of $CaCl_2$ at 37°C. The velocity constants of the mortality processes have been calculated from the well-known equation for an unimolecular chemical reaction:[2]

$$0.434\, K_1 = \frac{1}{t} \log \frac{a}{a - x} \qquad (1)$$

where a = the original number of viable bacteria
$a\text{-}x$ = the number of viable bacteria after time t

[2] Throughout this paper we have utilized the equations for chemical reactions which are direct and isolated, on the assumption that the changes which finally lead to loss of viability are irreversible. Cf. the theoretical treatment of the dynamics of reactions in homogeneous systems by Rice (1924).

t = time in hours
K_1 = velocity constant
log = logarithms to the base 10

Here we observe that the average value of K_1 is 0.232 and that the individual values of the velocity constant range between 0.213 and 0.256, with a probable error (P.E.) of ±0.011, or approximately 5 per cent.[3] For a biological experiment in which the errors of method involved in various of the procedures used in the technique of bacterial quantitation are appreciable, such a constancy of K_1 as is indicated by a P.E. of less than 5 per cent is

TABLE 1

The viability of Bact. coli in 0.145 M $CaCl_2$ solution

Experiment 37-C

INCUBATION PERIOD—t—HOURS	NUMBER OF VIABLE BACTERIA	LOGARITHM OF VIABLE BACTERIA	VELOCITY CONSTANT—K_1
0	15,200,000	7.182	
1.83	9,500,000	6.978	0.256
5.33	4,700,000	6.672	0.220
7.83	2,390,000	6.378	0.237
21.33	163,000	5.212	0.213

Average K_1 = 0.232
P. E. = ±0.011

striking. From the uniformity of the values of K_1 it follows that if the course of bacterial mortality is illustrated by a graph of logarithms of surviving organisms plotted against time the curve will approximate a straight line. That this is the case is evident from inspection of figure 1. There can be no doubt that such an experiment as this furnishes a general confirmation of the findings of Krönig and Paul, Madsen and Nyman, Chick, Cohen and others who have described the concordance between a bacterial mortality curve and the logarithmic straight line of the unimolecular mass action equation. It is also significant to observe that the mortality rate was comparatively low, approximately two-thirds of the bacteria having survived for a period of nearly two

[3] The probable error of the average was calculated on the basis: P. E. = $0.6745\sqrt{\Sigma d^2/n}$ where Σd^2 = the sum of the squares of the deviations from the average; and n = the number of values averaged.

hours. Experiments of this kind were specifically designed to meet the issue which has been raised by Loeb and Northrop (1917) and others that in the initial stages of a disinfection process, there is a lag period and that the logarithmic law is not followed if the rate of the process is measured in the early stages of the reaction.

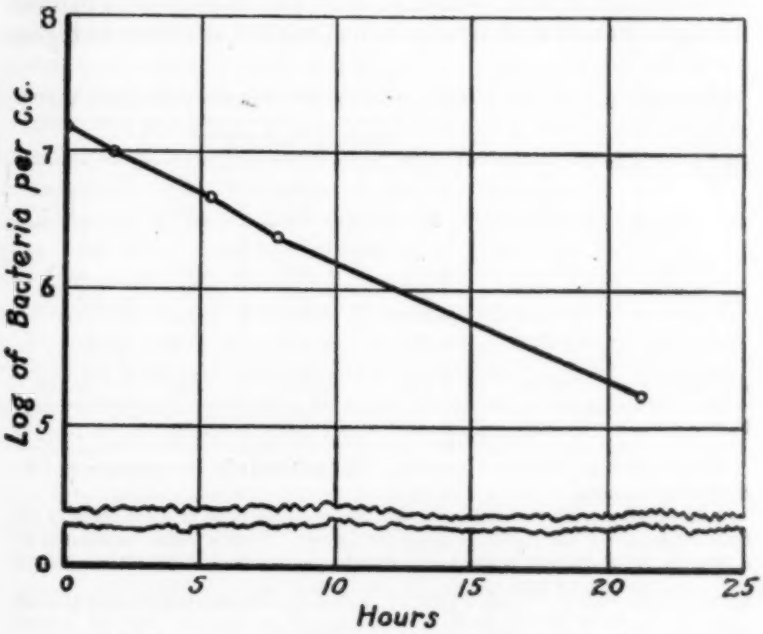

FIG. 1. BACT. COLI IN 0.145 M $CaCl_2$ SOLUTION AT 37°C.

In table 2 we have presented the results of typical experiments on the course of mortality in a bacterial population exposed to various concentrations of calcium chloride. (All of these tests were made at the same time, under similar conditions and with the same technique. Solution C and the data in column (4) are identical with experiment 37-C of table 1.)

From an examination of column (2) of the table it appears that in the presence of a very low concentration of calcium chloride

(0.00145 M) there was a slight multiplication of the bacteria.[4] It also appears that there was no multiplication of the bacteria in the first time interval (0 to 1.83 hours), an appreciable multiplication in the second interval (1.83 to 5.33 hours) a maximal multiplication in the third interval (5.33 to 7.83 hours) and a lesser multiplication in the final period (7.83 to 21.33 hours). From column (3) it appears that there was an even more marked multiplication of the bacteria in the presence of a ten-fold higher

TABLE 2

The viability of Bact. coli in $CaCl_2$ solutions of various concentrations at 37°C.

INCUBATION PERIOD—t-HOURS (1)	VELOCITY CONSTANTS (K_1) IN: A 0.00145M (2)	B 0.0145M (3)	C 0.145M (4)	D 0.725M (5)	E 1.450M (6)
0					
1.83	0	0.101	0.256	1.93	3.36
5.33	−0.178*	−0.384	0.220	1.07	1.93
7.83	−0.203	−0.636	0.237	1.14	∞†
21.33	−0.036	−0.037	0.213	0.766	....
Average.....	−0.104	−0.0239	0.232	1.23	2.65‡
P. E.....	±0.059	±0.199	±0.011	±0.43	

* A minus sign (−) before a value of K_1 indicates that the bacteria had *increased* in numbers.

† An infinity value (∞) indicates that the reaction went to completion in the time interval (i.e. no viable bacteria remaining). Such a value, calculated by setting $(a-x) = 0$ is inconsistent with the mass action law. Calculated values of K_1 will range from $\left(\frac{2.3}{t} \log a\right)$ to (∞) according as the bacterial count is taken between *1* and *o* per cc.

‡ Average calculated from the two finite values.

concentration of the salt (0.0145M), the rate of growth again attaining a maximal value in the third interval.[5] Columns (4),

[4] It is recognized that the application of the unimolecular equation to the tests in which there are observed *increases* in the number of viable bacteria is not strictly sound. It is carried out for the sake of uniformity of procedure and in order to provide in the $(-K_1)$ values a convenient measure of the rate of multiplication.

[5] The stimulating effect at this salt concentration was unusual. As will be shown later on a concentration of 0.01 M $CaCl_2$ generally proves slightly toxic.

(5) and (6) representing data from viability experiments with solutions of higher salt concentrations (0.145M, 0.725M and 1.450M) show no multiplication of the bacteria, but, instead, progressively higher mortality rates.

It would appear, then, from these data that in very dilute aqueous solutions $CaCl_2$ stimulates *Bact. coli* to multiply and that in higher concentrations it is highly toxic. Now, it is especially significant to observe that in one of the three toxic solutions (column (4)) the course of mortality follows that of an unimolecular reaction, witness the sensible constancy of the values of K_1. If only this particular concentration of $CaCl_2$ had been studied and had been presented alone as in table 1, it would have implied a confirmation of the unimolecular disinfection theory. However, examination of the last two columns of table 2 brings out clearly that with more toxic concentrations of $CaCl_2$ the values of K_1 are not even approximately constant. They show, instead, values which decline steadily for successive intervals of time. If the constancy of K_1 in solution C means that the reaction between $CaCl_2$ and *Bact. coli* is essentially unimolecular,[6] it becomes impossible to reconcile the data in column (4) with those in columns (5) and (6) without making additional assumptions.

If the course of a disinfection process be such as to give the logarithmic straight line of an unimolecular reaction and if it be assumed that the fundamental reaction is unimolecular, we might consider that of the reacting agents (1) disinfectant, and (2) living cell, one or the other is present in excess and that its concentration is not changing significantly during the course of the reaction. Inasmuch as the constant K_1 obviously changes with a change in salt concentration it must be assumed that the reacting substances of the bacterial cell (whether those cells are dead or alive) must always be present in excess if anything approaching the monomolecular curve is to be attained.

[6] Assuming that one of the reacting agents is present in excess and that hence its concentration remains essentially constant. In such a case, the reaction may really be of a higher order, but it will *appear* to be of the first order.

IV. DEVIATIONS FROM THE LOGARITHMIC CURVE

We may now proceed to consider the deviations from a regular logarithmic curve which are manifest on a closer analysis of the results of such experiments as are under discussion. Almost all the observers who have studied this problem report such deviations. At the beginning of the observation period marked and more or less irregular mortalities are manifest and during the later course of an experiment there is a rather regular tendency for the rate of dying to become progressively less. Even in the work of Chick this tendency is clearly manifest.

TABLE 3

Viability of Bact. coli in distilled water

INCUBATION PERIOD—t-HOURS	VELOCITY CONSTANTS (K_1) AT pH = :						
	4.0	5.0	6.0	6.5	7.0	7.5	8.0
0							
1	0.14	0.14	0.18	0.09	0.39	0.25	0.23
3	0.31	0.11	0.10	0.14	0.21	0.47	0.21
6	0.54	0.12	0.07	0.20	0.24	0.42	0.35
9	0.51	0.04	0.02	0.29	0.17	0.34	0.23
24	(*)	0.12	0.01	0.16	0.06	0.15	0.10
Average K_1	0.37	0.11	0.08	0.18	0.21	0.33	0.23
Number of experiments	2	2	4	2	10	2	4

* See table 2, footnote †, for explanation.

In all viability studies there are marked irregularities, such as we are accustomed to find in biological investigations,—which make the elucidation of the more subtle influences at work exceedingly difficult. The only way to overcome such difficulties is by the use of averages obtained from the results of large numbers of experiments, and we have therefore attempted to throw some light upon the problem in hand by working out such averages for a considerable number of experiments. These data are presented herewith in tables 3 to 5 and figures 2 and 3. In these tables the values for K_1 have been expressed only in two decimal places since

the chance errors are such as to rob the third decimal of any practical significance.[7]

From tables 3 to 5 it is obvious that there is a rough general approximation to a constancy of K_1 with a given concentration of a given toxic substance. In distilled water solutions near

TABLE 4

Average reaction velocities of mortality of Bact. coli in various concentrations of $CaCl_2$, and after various periods of exposure

CONCENTRATION $CaCl_2$ NORMALITY	NUMBER OF TESTS	HOURS					
		1	3	6	9	24	Average
0.001–0.002	1–4	−0.18	0.04	−0.10	−0.05	−0.02	−0.06
0.01–0.03	28–31	0.32	0.10	0.09	0.05	0.01	0.114
0.07–0.14	20–24	0.16	0.18	0.17	0.12	0.09	0.144
0.4	2–11	0.84	0.80	0.73	0.60	0.48	0.690
0.7	3–15	1.30	1.34	1.01	1.17	0.82	1.128

TABLE 5

Average reaction velocities (K_1) of mortality of Bact. coli in various concentrations of NaCl and after various periods of exposure

CONCENTRATION NaCl NORMALITY	NUMBER OF TESTS	HOURS					
		1	3	6	9	24	Average
0.01–0.03	7–8	0.00	0.00	−0.03	−0.02	−0.02	−0.002
0.07–0.14	9–11	0.11	0.09	0.10	0.09	0.08	0.094
0.4	6–8	−0.05	0.14	0.17	0.24	0.03	0.106
0.7	19–20	0.04	0.24	0.24	0.22	0.23	0.194
0.9–1.0	14–16	0.12	0.34	0.29	0.20	0.09	0.201
1.4	5–15	0.44	0.71	0.81	1.22	0.48	0.732

neutrality and in dilute salt solutions K_1 is very small, that is the rate of bacterial mortality is low. A very dilute solution of either NaCl or $CaCl_2$ is definitely favorable to survival, causing in general an actual increase in numbers ($-K_1$). With an acid concentration equivalent to pH 4.0 or an alkaline concentration equivalent to pH 7.5 the mortality constant increases as it does

[7] The averages have been obtained arithmetically,—a procedure not perhaps justified on mathematical grounds, since we are dealing with logarithmic terms, but indicating general trends with an accuracy which has been found by calculation to be sufficient for the present discussion.

with NaCl concentrations of 1.0 M or over and with a $CaCl_2$ concentration of 0.4 M and over.

Closer analysis of the figures shows, however, rather definite deviations from a constant value of K_1. Even with the distilled water data in table 3 it is apparent that the K_1 values are generally

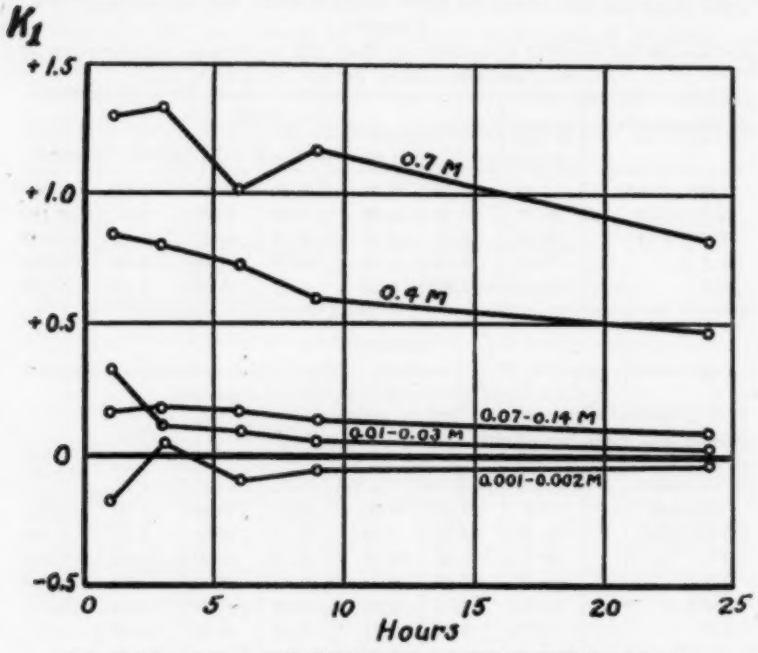

FIG. 2. BACT. COLI IN $CaCl_2$ SOLUTIONS OF VARIOUS MOLARITIES

lower after twenty-four hours than at earlier periods. This fact is brought out much more clearly by the averages based on more abundant data in tables 4 and 5 and figures 2 and 3.

In the $CaCl_2$ solutions (see fig. 2) every one of the five curves shows an almost steady decrease in K_1 with the progress of the experiment. In the NaCl solutions (fig. 3) there is apparently an increase in K_1 during the first few hours with a subsequent decrease. In the 1.4 M concentration the variation is so great as to eliminate almost all semblance to the curve of a monomolecu-

lar reaction. This may in part be due to the fact that while the averages for one, three and six hours are based on 15 to 16 observations, those for nine and twenty-four hours are based only on 5 observations. Within the series of 5 experiments carried through the whole twenty-four hours the same relation is, however, manifest; the average K_1 values for these five experiments

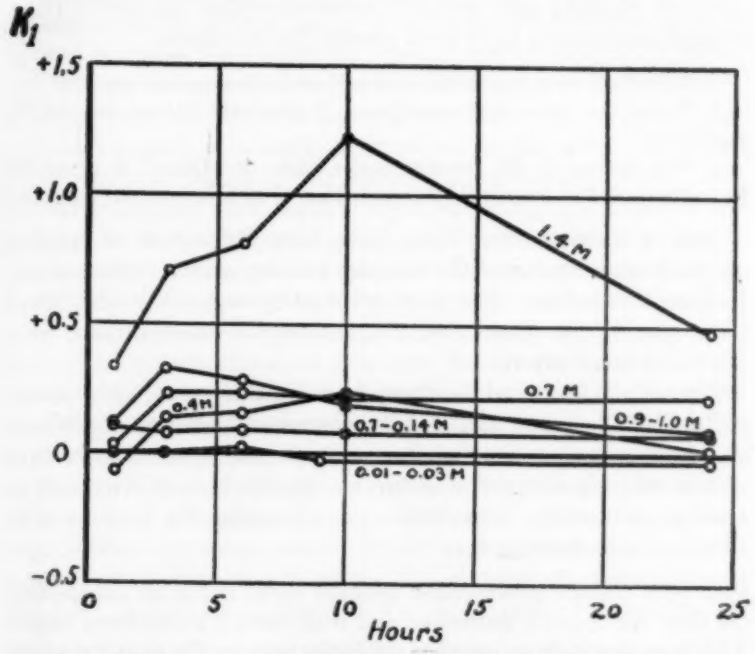

FIG. 3. BACT. COLI IN $NaCl_2$ SOLUTIONS OF VARIOUS MOLARITIES

being as follows: 1 hour, 0.37; 3 hours, 0.57; 6 hours, 0.88; 9 hours, 1.22; 24 hours, 0.48. It appears to us probable that there is a real increase in K_1 values during the first few hours of exposure to NaCl solutions; and there is certainly a progressive decrease in K_1 during the later hours in most of the solutions studied.

V. CRITICISMS OF THE VIEW THAT MORTALITY FOLLOWS A LOGARITHMIC CURVE

Most of the investigators who have studied the dynamics of disinfection,—Krönig and Paul (1897), Ikéda (1897), Madsen and Nyman (1907), Chick (1908) and Cohen (1922),—have been chiefly impressed with the broad general similarity between the curve of viability and that of a monomolecular reaction. Their general conclusions have been essentially as follows:

a. Disinfection is a gradual and not an instantaneous process.

b. Under the prescribed conditions, it generally follows an orderly sequence.

c. The course of the process under these conditions is generally logarithmic and is described by the equation of an unimolecular reaction.

Among these workers there have been differences of opinion on the interpretation of the findings but not on the nature of the findings themselves. Three other investigators on the other hand have specifically questioned the validity of conclusions *b* or *c* (or both) listed above.

First of all, Loeb and Northrop found in their work with Drosophila (fruit fly) that the mortality curve at higher temperatures simulates a probability distribution curve and is not of the nature of a mass action logarithmic curve. As has been pointed out in another connection, these authors in discussing the work of Miss Chick, overlooked the fact

> that Miss Chick's unimolecular reaction curve is not an assumption (as they state) but a deduction, and that there is no inherent reason why there should be an essential similarity between the equation which describes the rate of mortality of bacteria subjected to a large excess of toxic substances and the rate of mortality of fruit flies whose span of life—according to Loeb and Northrop—is probably limited by the production within the animal, in the course of its own metabolism, "of a substance leading to old age and natural death or by the destruction of a substance or substances which normally prevent old age and natural death" (Falk, 1923, pp. 95–96).

It appears, also, that there may be but scant validity in the comparison of mortality data for *Drosophila melanogaster*—an

animal form which shows bisexuality and differentiation between soma and germ plasm, and bacteria—plant forms in which neither sexual nor somatic specilizations are demonstrable.

The second of these critics of the monomolecular reaction hypothesis, Brooks (1918) showed that if erythrocytes are suspended in an indifferent medium and are subjected to a hemolytic agent (radiation from a mercury vapor arc or specific hemolytic antibody) the curve of liberated hemoglobin plotted against sampling time gives not the curve of a mass action reaction but of an asymmetrical sigmoid, i.e., showing an initially slow rate of hemolysis, an increasing rate to a maximum, a gradual retardation and the attainment of an equilibrium. Without going into the details of his arguments, we may indicate that Brooks interprets this finding by postulating the existence of differences in resistance of the cells to the hemolytic agent. Brooks further distinguishes between the resistances of the cells to the hemolytic agent which determine the "course of the process" (i.e., the time curve) and the "fundamental reaction" (i.e., "the physicochemical processes in the protoplasm") and treats each as an independent variable. Hence, he concludes that the time curve cannot directly indicate the nature or order of the fundamental reaction.

Finally, Fulmer and Buchanan (1923) have reported the results of a series of experiments on the toxicity of phenol and phenol and alcohol for yeast cells. Their measurements of toxicity were made by staining periodic aliquot portions taken from a yeast cell suspension exposed to the toxic agent. They say:

> It has been shown that all cells which take up the stain are dead, that is, they no longer can reproduce. However, it was found that the ability to reproduce was lost *before* the cell acquired the ability to stain. In other words, cells which stain are dead, but a dead cell does not of necessity immediately acquire the ability to stain. The two phenomena are closely related, so that cell staining is a satisfactory criterion of toxicity (p. 79).

Of twenty-seven sets of experiments which they cite, they find that in four there was no period with a maximal rate of dying,

in nineteen there was one and that in four there were two well marked maximal periods. They conclude, therefore, that their time curves do not in general prove to be logarithmic; that such resemblances as have been found between such time curves and unimolecular or logarithmic curves are superficial and fortuitous; and that variations in resistance of individual cells and the distribution of such variations must be regarded as of fundamental importance in accounting for rates of death of microörganisms.

The finding of Fulmer and Buchanan that the time curves of their yeast disinfections are not even in a general way logarithmic, and their conclusion that such resemblances as have been found are "superficial and fortuitous" demands critical evaluation.

It is recognized that staining with dilute methylene blue may be—as Fulmer and Buchanan state—an adequate test for the mortality of the cell. It must also be recognized, however, that such a technique may serve as an adequate criterion of completed toxicity, i.e., of lethality, without providing a criterion for measurement of *rate* of lethality, unless it be first demonstrated that the time interval which must elapse after the death of a cell before it displays avidity for the stain is (1) constant for all cells in a test suspension, (2) is invariate with different toxic agents, and (3) is sensibly small by comparison with the time requisite for the effective action of the toxic agent. The reasons for conditions (1) and (2) are self evident. Condition (3) arises out of the consideration that—assuming fulfillment of (1) and (2)—the rate curve would show anomalous inflections, especially at the beginning and end, if the time for development of avidity for the stain is appreciable by comparison with the time necessary for the disinfectant to exert toxicity. Thus, if the first interval of time on a lethality rate curve were equal to the time which elapses between the establishment of the status which is measured by loss of reproductive capacity and the development of avidity for the dye, the rate of dying in this first period—as measured by the staining reaction—would be zero. In the next interval of time it would be something greater than zero,—in the direction of a positive value. In successive intervals the rate would increase and approximate the true rate of dying. As the end of the dis-

infection was approached, the rate would in a similar manner decline from the true value for the rate of mortality. Fulmer and Buchanan picture curves of just the kind which could be deduced from this analysis. (A precise, mathematical treatment of consecutive reactions is presented by Mellor, 1904, pp. 113 ff. and is reconsidered in its biological applications by Osterhout, 1922.) It is also pertinent to note that if initial and final lag deviations from a logarithmic straight line are attributable to the catenary nature of the fundamental reactions involved in the development of dye avidity by yeast cells, there should be no inflection in the direction of terminally accelerated rates. Nor are any clearly marked inflections of this kind observed by Fulmer and Buchanan.

VI. CONCLUSIONS IN REGARD TO THE REGULARITY OF DISINFECTION PROCESSES

It appears from a general review of the subject that while Fulmer and Buchanan find no regularity in the disinfection process as measured by the stainability of yeast cells, all of the other workers with microbic viability report a general correspondence with the logarithmic curve but with more or less distinct deviations toward the beginning and the end of the process. Cohen's curves are, on the whole, the most regular with which we are familiar. Even in his studies, however, many of the curves for mortality at 30° show a lessened rate of reduction toward the end. Chick's figures reveal much greater deviations from the monomolecular straight line, although she is inclined to minimize them in her discussion. Our own studies, as pointed out above, show a marked and definite tendency toward a decreasing rate for K_1 as the process of disinfection continues.

It seems, in general, that while the rate of disinfection follows a logarithmic curve it does not, as a rule, follow it strictly; and the degree of deviation from a monomolecular reaction varies with the particular type of disinfection which is going on. The death of bacteria in water at low temperature follows the monomolecular curve very closely (Cohen); the destruction of bacteria by standard disinfectants (Chick) deviates slightly from it; the mortality

in presence of NaCl and $CaCl_2$ (our own data) shows greater differences; the mortality of Drosophila (Loeb and Northrop) and the hemolysis of red blood cells (Brooks) differ still more; while the stainability of yeast cells after exposure to phenol and alcohol exhibits very little correspondence with the monomolecular law. It appears to us that this is exactly what might be expected, since it seems inherently very improbable that all these various processes destructive of protoplasm should be of the same simple chemical nature.

VII. POSSIBLE EXPLANATIONS OF DEVIATIONS FROM THE LOGARITHMIC CURVE

Where deviations from the logarithmic curve are actually present, we are by no means convinced that the assumption of variability on the part of the reacting cells is a necessary or an altogether satisfactory one. It is significant to recall that "resistance" of a cell to a lytic or to a lethal agent is not an independently *measurable* factor. "Resistance" is known only as a function of time. Thus, a cell is "more resistant" to a lytic or a disinfectant agent if a longer period—and is "less resistant" if a shorter period —of time must elapse before it is lysed or killed. We are not aware that "resistance" is known in any other terms or is measurable in any other units. It represents, therefore, a state whose existence is *assumed* but not *proved*.

In the field of chemical phenomena it is not clear why molecules, atoms or ions which eventually take part in a reaction do not all react to the completion or equilibrium of the reaction in a single unit of time.[8] In the terms of the kinetic theory, it was customary to consider that the interactions were determined by the occurrences of collisions or bombardments and that these were determined by chance (that is, by the operation of a large number of factors), and by the nature and the concentrations of each kind of substance. On this ground, however, it became difficult to account for the fact that the temperature coefficients of most chemical reactions are much greater than postulates of the ki-

[8] Cf. Rice (1924, p. 898, et seq.).

netic theory would warrant. In terms of the quantum theory, as Cohen (1923) has indicated, the explanation for the time curve would lie, presumably, with the chance distribution of variable energy quotas in the molecules of a substance. For the time curve of a disinfection process, Chick (1908) was unable to conceive the existence of such differences in the "resistance" of the cells to the disinfectant as could account for its shape. She considered instead, from analogy with the dynamics of heat coagulation of proteins, which closely resemble the destruction of bacteria by hot water, that "an explanation has been sought in temporary changes in the energy of the molecules, as a consequence of which all molecules do not possess the same sensibility to attack at the same moment. Some such property is therefore attributed to the molecules (or aggregates of molecules) of the constituent protein of the bacteria, whereby at a given moment only a certain proportion is liable to attack; the amount being dependent upon the concentration at the moment of unaltered protein, in other words the number of bacteria surviving in unit volume."[9] It is at once evident that these views are essentially the same as those deduced from general chemical or thermodynamical considerations. From this standpoint while the curve for an array of bacteria of uniform chemical composition should show a monomolecular curve of mortality, a population of variable composition (resistance) might be expected to yield a curve made up of several different logarithmic curves, each component curve corresponding to a different degree of resistance.

Some years ago, we pointed out (Winslow and Falk, 1920) that the postulation of such a specific resistance factor was unnecessary and that it needlessly complicated the concepts of disinfection dynamics. We also pointed out that the results obtained from quantitative experiments can be explained if it be assumed that the death of a cell be due not to the results of a single

[9] Similarly, Arrhenius (1915) had clearly recognized the discrepancy between a postulation of different resistances in a bacterial population and the observation of a logarithmic course in disinfection. He concluded that "the different lifetime of the different bacteria does not, therefore, depend in a sensible degree on their different ability to resist the destructive action of the poison" (pp. 78–79).

reaction but to a chain of reactions (i.e., conceiving the fundamental reaction of Brooks as a catenary reaction). Brooks himself considered that "the fundamental reaction may be either a simple process, or the expression of a complex series of changes whose rate is at all times governed by that of the slowest of the series" (p. 79).

In the field of chemical dynamics it is known that a reaction between reagents may be of one order with one set of concentrations and of another with a second. Thus it appears that the reaction between potassium ferricyanide and potassium iodide works out to be unimolecular with respect to the former component, whereas at other concentrations it is shown to be bimolecular (Mellor, 1904, p. 64; also, Rice, 1924, p. 883). In table 6 we cite the results of our Experiment 47-B on the course of mortality for *Bact. coli* in 0.145 $CaCl_2$ solution, showing the velocity constants (K_1, K_2, K_3, and K_4) which were calculated from the following equations for uni-, bi-, tri-, and quadri-molecular reactions:[10]

$$K_1 = \frac{2.3}{t} \log \frac{a}{a - x} \tag{2}$$

$$K_2 = \frac{1}{t} \frac{x}{a(a - x)} \tag{3}$$

$$K_3 = \frac{1}{t} \frac{x(2a - x)}{2a^2 (a - x)^2} \tag{4}$$

$$K_4 = \frac{1}{t} \frac{1}{3} \left(\frac{1}{(a - x)^3} - \frac{1}{a^3} \right) \tag{5}$$

From column (2) it appears at once that the disinfection did not follow the logarithmic course illustrated in a similar experiment cited in table 1 (the same concentration of $CaCl_2$ was used in the two cases) as is evident from the inconstancy of K_1. The steadily decreasing values of K_1 indicate that the reaction between disinfectant and viable cell was proceeding at a rate higher than that

[10] It should be clearly understood that these equations represent only first approximations for the present purposes. The actual concentrations of the two, three or four reacting molecules cannot be determined and are therefore taken as equal merely for the sake of making these first approximations.

indicated by the so-called logarithmic law. Further, if the first and last two values of K_2 (column 3) be disregarded, the reaction may be considered to have proceeded in accordance with the course of a bimolecular chemical reaction. The approximately constant values of K_3 in column (4) suggest, however, that after the first three or six hours, the reaction proceeded in the manner of a chemical reaction of the *third* order. These observations are confirmed by the progressively increasing values of K_4, listed in the last column, which indicate that the course of the disinfection rate was lower than that postulated from the equation of a *fourth* order reaction. In brief, then, the data of table 6 indicate that when the disinfection of *Bact. coli* by 0.145 M $CaCl_2$ does not fol-

TABLE 6

The viability of Bact. coli in 0.145 M $CaCl_2$ solution at 37°C.

INCUBATION PERIOD—HOURS (1)	VELOCITY CONSTANTS			
	K_1 (2)	K_2 ($\times 10^3$) (3)	K_3 ($\times 10^{10}$) (4)	K_4 ($\times 10^{16}$) (5)
1	1.56	2.14	3.39	6.50
3	0.65	1.16	2.59	7.01
6	0.46	1.39	6.48	39.3
9	0.34	1.30	8.23	68.9
12	0.27	1.17	8.50	83.9
24	0.15	0.85	8.92	127.0
125	0.034	0.36	8.00	236.0

low the logarithmic course of a unimolecular reaction in a particular experiment, it may be following the course of a reaction of a higher order through a considerable period of time (i.e., *second* order, 1 to 12 hours; *third* order, 9 to 125 hours in table 6). Further, we may even consider that by the method of best fit of the equations the course of disinfection in experiment 47-B was chiefly that of a third order chemical reaction with a lag in the early stages which approximated the course of a second order reaction.

We come now to the consideration of a phase of disinfection theory which is suggested by our experimental findings and which hitherto appears to have been neglected. More than thirty

years ago, Richet (1892) pointed out that for each of a considerable series of salts whose influences on lactic fermentation of milk whey were studied it is possible to demonstrate that there are concentrations which are:

a. Indifferent
b. Stimulating
c. Inhibitive
d. Toxic

His series included salts of Na, K, Li, Mg, Ca, Sr, Ba, Fe, Pb, Zn, U, Al, Cu, Hg, Au, Pt, Cd, Co, and Ni. His findings, especially with respect to the existence of the stimulating concentrations, are the more noteworthy because of the inclusion in his series of salts with such highly toxic cations as those of cadmium, cobalt, nickel, copper, gold, platinum and mercury. Recently, Dr. Hotchkiss (1923), unaware of Richet's work, came to essentially the same conclusions from more carefully conducted experiments on the stimulating and inhibiting influences of a series of salts upon the growth of *Bact. coli*.[11] She was able to find for 15 of 23 chlorides studied a concentration which stimulated growth—as indicated by the production of a turbidity greater than that in the control cultures to which no salt had been added. The stimulating salts in her series included not only chlorides of K, Na, NH_3, Li, Sr, Mg, Ca and Ba, but also of such toxic metals as Ti, Sn, Ni, Pb, Co and Hg; and she was persuaded that stimulating concentrations for the other eight salts could have been established by more exhaustive study. Madsen and his associates have recenty reported[12] similar stimulating effects of minute amounts of Cu and Mn upon the growth of the tubercle bacillus.

Accepting these conclusions as indicative of a general biological principle,[13] it is of considerable interest to reëxamine from this point of view the course of mortality of *Bact coli* in the presence of various concentrations of calcium chloride. In a more detailed report (Winslow and Falk, 1923a) and in tables 4 and 5

[11] The same strain as that used in our studies.
[12] Meeting, Laboratory Section, A.P.H.A., Detroit, November, 1924.
[13] Further evidence is cited at length in a review by Falk (1923).

we have indicated that either sodium chloride or calcium chloride, in suitable concentrations, may be stimulating as well as toxic to *Bact. coli* suspended in non-nutrient menstrua. If from our data average velocity constants of mortality (K_1) are calculated (assuming, tentatively, the applicability of the unimolecular reaction equation), we obtain (see last columns of tables 4 and 5) a series of values which pass from negative to positive values and which give a reasonably smooth curve for both salts (fig. 4). We are not aware that any chemical theory of disinfection which has previously been presented is in harmony with the fact that these curves are approximately continuous, above and below the

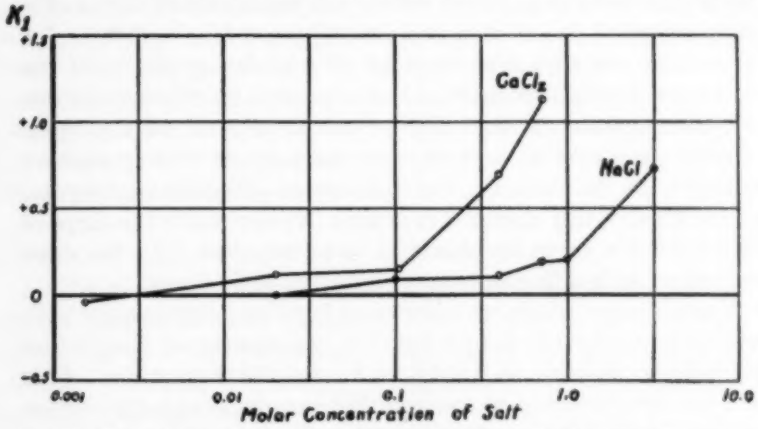

FIG. 4. VIABILITY OF BACT. COLI IN SODIUM AND CALCIUM CHLORIDE SOLUTIONS

base line. We can scarcely assume that the velocity of a lethal reaction is inhibited by certain concentrations and accelerated by other concentrations of one of the reacting substances. We are not aware that chemical dynamics of homogeneous systems can be harmonized with such conditions. The curve, presumably with an origin when both x and y are 0,[14] suggests smooth and continuous gradations for values of K_1 from 0, through negative to progressively increasing positive values. It therefore implies

[14] Because in pure water (x = 0) the bacteria maintain themselves in practically undiminished numbers for twenty-four hours.

that there is no sharp discontinuity between such effects of a reagent as result in acceleration of the reproductive rate to such as markedly accelerate the rate of mortality. It might be assumed that the effect of a toxic agent on *Bact. coli* becomes manifest because mortality is the consequence of a stimulation of reproduction to a degree which is incompatible with maintenance of viability (i.e., if the fundamental reactions of stimulation and toxicity are identical); but such a view would be extremely far-fetched except perhaps in connection with the influence of temperature. If it be considered that this assumption is unwarranted, it becomes necessary to consider that the action of a toxic substance upon a bacterial cell is not explicable in terms of a simple type of chemical reaction.[15] The course of a disinfection process is the algebraic resultant of stimulating effects of the toxic agent upon reproduction and of truly toxic effects measured by accelerations of mortality rates. Considered in this light, the time curve of a mortality process may lie at any position between the extremes, i.e., the time curves of the stimulating and of the intoxicating chemical reactions. Variations in the shape of this resultant curve are therefore to be expected with the same as well as with different concentrations of toxic agent.

Certain data which we have cited from our experiments indicate a general concordance between the courses of disinfection by calcium chloride and of bi- or tri-molecular reactions. It is not our aim, however, to contend that the course of a disinfection process necessarily or even generally simulates that of a bi- or tri-molecular chemical reaction. We wish merely to emphasize

[15] By making additional assumptions, especially by assuming reaction in a heterogeneous system in which the number of phases is variable, it may be possible to deduce the equation of such a curve. If the disinfection of a bacterial suspension by $CaCl_2$ is treated as a reaction in a heterogeneous instead of a homogeneous system and if the course of the process is found in accord with the logarithmic law, the significance of the finding becomes radically different from that which has been generally placed upon it. It may mean that in the changes which lead to loss of viability a number of reactions (physical or chemical or both) are involved and that the slowest (and determining) one may be of the nature of a diffusion reaction or of any one of many kinds of multi- or inter-phase reactions (vide Taylor, 1924). The precise treatment of these conditions cannot be conducted with our data.

that although disinfection processes generally give the logarithmic curve and are approximately described by the equation of a unimolecular reaction, under certain conditions—particularly in the presence of certain mild disinfectants—the course of the process may more closely approximate that of a multimolecular reaction.[16]

In the present state of knowledge, the time curve of a disinfection process remains as a valuable tool in the measurement of toxicity but not as a guide to the chemistry or physics of the fundamental reaction between cell or cell constituents and toxic agent. Conclusions to the effect that the time curve is always, necessarily, logarithmic appear not to be in accord with experimental findings. The exceptions from the logarithmic course which have been reported here lead to the conclusion that the mechanism of a disinfection process is probably highly complex. On the other hand the observed variation can be quite plausibly explained on chemical grounds without the introduction of the assumption of biological variability.

VIII. GENERAL CONCLUSIONS

1. The mortality of *Bact. coli* in solutions of NaCl and $CaCl_2$ and in distilled water of varying pH follows a generally logarithmic course and may be roughly described by the equation of a unimolecular chemical reaction, as has been shown to be the case for other processes of disinfection.

2. This relationship is not, however, a close or an exact one. Like most other students of this problem, we find, on close analysis, more or less marked deviations from the logarithmic curve. With NaCl the rate of reaction seems to increase at first. With both salts, and with acid and alkali, it gradually decreases as the experiment proceeds through its later stages.

3. Where disinfection does not follow a logarithmic course and is not to be described by the unimolecular equation, the course of the process may sometimes be described by the equation of a

[16] It is significant to recall that from a recalculation of Chick's data on phenol disinfection, Watson (1909) concluded that the reaction was of at least the seventh order.

bi-, tri-, or higher multimolecular reaction. Such an explanation may render unnecessary the assumption of variability in biological resistance to account for deviation from a logarithmic mortality curve.

4. When used in sufficiently low concentration, NaCl and $CaCl_2$ may be not only without toxic properties for *Bact. coli*, but may even stimulate the organisms to increased growth and reproduction. This observation is in harmony with the conclusions of Richet, Hotchkiss, Madsen and others and appears to be an illustration of a general biological principle. For a series of concentrations of $CaCl_2$ acting upon suspensions of *Bact. coli* a curve may be plotted which shows continuous gradations between intoxicating and stimulating effects.

5. From an analysis of the time curves of disinfection processes it appears that the reaction between disinfectant and cell or cell constituents is probably highly complex; and that while the logarithmic curve best expresses its general rate, the correspondence is not, and cannot be expected to be, always a close one.

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VIABILITY OF BACTERIUM TYPHOSUM IN ICE CREAM

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The experimental results here reported were obtained in connection with a study on the effect of carbon dioxid on typhoid bacteria in ice cream. After the desired information on this point was secured, the ice cream was kept for the purpose of determining how long the typhoid germs would survive in ice cream.

One gallon of ice cream mix was prepared, which contained 12 per cent sugar, 12 per cent solids other than fat, and 10 per cent fat. The mix was sterilized, inoculated with *Bacterium typhosum*, and then incubated until the bacterial count was about 25,000,000 per cubic centimeter. It was then frozen and immediately stored in the hardening room. The temperature in this room fluctuated between 8 degrees below zero and 8 degrees above, but for the most part was about $-4°$F. The ice cream was prepared on January 11, 1922, and was held under the above conditions until all the ice cream was used up by sampling, the last sample being taken on May 20, 1924. Samples for bacteriological examination were taken from the mix just before it was frozen, from the freshly frozen cream, and then at varying intervals during the two years and four months of storage. The bacterial counts are recorded in table 1.

The original culture, as well as the cultures which were recovered from each sample of the ice cream were subjected to agglutination and other laboratory diagnostic tests. These cultures were also tested for us by Mr. Luther Thompson. The culture obtained from the last sample, when the ice cream was two years and four months old, was tested by the authors, by Mr. Thomp-

son and also by Prof. H. M. Weeter.[1] Positive *Bacterium typhosum* tests were obtained in all cases.

Just before freezing the mix gave a count of 25,000,000 bacteria per cubic centimeter. About an hour after freezing, the ice cream gave a count of 51,000,000 per cubic centimeter. This apparent increase was probably due to the breaking up of the bacterial clumps. After five days of storage the count was reduced about 90 per cent and in twenty days the reduction was more than 95 per cent. In 134 days approximately one germ out of every two hundred survived. At the end of one year about one germ out of every thousand survived, and at the end of two years one germ out of every ten thousand survived. After two years and four months of storage living typhoid germs were still

TABLE 1

Persistence of Bacterium typhosum in ice cream

SAMPLES TAKEN	TYPHOID BACTERIA PER CUBIC CENTIMETER OF ICE CREAM
Before freezing	25,000,000
Freshly frozen	51,000,000
5 days old	10,000,000
12 days old	7,000,000
20 days old	2,200,000
70 days old	660,000
104 days old	900,000
134 days old	210,000
165 days old	640,000
170 days old	711,000
200 days old	60,000
260 days old	57,000
290 days old	53,000
342 days old	51,000
430 days old	30,000
544 days old	13,000
648 days old	11,000
2 years	6,300
2 years, 4 months	Living typhoid germs present

[1] We are greatly indebted for the tests to Mr. Luther Thompson, formerly of the University of Illinois, now with Mayo Brothers, Rochester, Minnesota, and to Professor H. M. Weeter, Medical School, Louisville, Kentucky.

present. Accurate count at this time was impossible on account of contamination. It will be observed in table 1 that the samples taken when the ice cream had been in storage for 165 days gave higher counts than the previous samples. To check this point, another set of samples was taken five days later which again gave similar counts. An inquiry brought out the fact that one of the attendants had removed the experimental ice cream a few days before to an adjoining room for an hour and one-half. This room had a temperature of 40°F. The ice cream did not melt. Whether there was any multiplication of the germs at this time could not be determined.

The counts given in the accompanying table indicate that there was a continual decrease in the number of the typhoid germs. The decrease was rapid at first but gradually slowed down. A small percentage of the germs were able to withstand the adverse conditions for a long time. The experiment had to be terminated at the end of two years and four months because all the ice cream was used up by sampling. Some of the typhoid germs were still living at this time and there is every reason to believe that they would have survived even longer.

It is evident that low temperature such as is used for the storage of ice cream can not be relied upon to destroy typhoid germs in ice cream. This had already been demonstrated by others (1).

Why some of the typhoid germs were able to survive so much longer than others is an interesting question still open for further investigation. From other data not given here it was found that the nature of the medium in which the germs are kept is a contributing factor.

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A NOTE ON THE SPIROCHAETES OF TERMITES

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The original observations of spiral organisms in the intestines of termites or "white ants" were made by Leidy (1877), who noted that the gut of *Reticulitermes flavipes* (*"Termes flavipes"*) was distended with material consisting largely of infusorial and other parasites. Of these parasitic forms, Leidy identified three as animal and two as vegetable in character. One of these latter he termed a spirillum, "probably *Spirillum Undula.*"

In a later study of the parasites of termites, Leidy (1881) called the spiral organisms "vibrios" and declared they most nearly resembled the *Vibrio serpens* of Müller. Leidy gave these spiral forms the name *Vibrio termites* and described them as rectilinear and regularly undulant with 3 to 6 waves, commonly stationary and undulating more or less rapidly, but often advancing or receding with variable rapidity, sometimes becoming quiescent. Occasionally they were observed to bend at an obtuse angle while continuing to undulate. The smallest individuals were straight but in motion became bent in the segment of a circle; not infrequently they adhered together by one end, thus forming radiating groups.

Other observations on spiral organisms in termites have been reported by Grassi and Sandias (1893–1894), who recorded spirilla in European termites—*Kalotermes flavicollis* and *Reticulitermes lucifugus* (*"Termes lucifugus"*).

The latest report on organisms of this type in termites has been made by Dobell (1910–1913), who noted them in the course of certain studies on parasitic protozoa from Ceylon.

To these spiral organisms Dobell gave the name *Spirochaeta termitis*, Leidy, as he concluded that the vibrios described by the earlier worker were undoubtedly spirochaetes or treponemata. Dobell observed in his termites two types of spiral organisms, one of which was much larger than the other and the description of which is identical with the *Treponema minei* of Prowazek. These organisms Dobell reported as varying from 20 to 60 micra long by 0.5 micron in width, pointed at both ends, possessing no flagella or crista, and actively motile. When stained by Giemsa stain these organisms take a uniform pink and occasionally exhibit a granular structure.

With these observations in mind, it occurred to the author that it might be of interest to examine other species of termites, as a considerable number of species were immediately available in this laboratory. In this way it was hoped to get a better idea as to the distribution of spiral organisms among the various families of termites and to study more carefully the morphology of such organisms as might be observed. To do this it appeared necessary to examine fresh preparations, made by emulsifying the material contained in the gut of the termites in a saline solution, by dark field illumination and to devise a method of staining that would permit careful study of smear preparations of the same material.

FIG. 1. A REPRESENTATIVE FIELD OF A SMEAR PREPARATION MADE FROM THE STOMACH CONTENTS OF NASUTITERMES MORIO

A similar abundance of spirochaetes will be seen in smears made from all the species examined.

FIG. 2. A LIMITED FIELD OF A PREPARATION OF TERMOPSIS NEVADENSIS

Note the extreme size of some of the spirochaetes seen in this species

FIG. 3. ANOTHER PREPARATION OF TERMOPSIS NEVADENSIS SHOWING THE SMALLER TYPE OF ORGANISM

FIG. 4. ILLUSTRATION OF THE HEAVILY STAINING TYPE OF ORGANISM FOUND IN CRYPTOTERMES BREVIS

FIG. 5. REPRESENTATIVES OF THE TYPE OF SPIROCHAETAE SEEN ONLY IN KALOTERMES SCHWARZI

Note the occurrence of the circular coils

FIG. 6. ANOTHER FIELD FROM THE SAME PREPARATION AS THAT USED IN THE PRECEDING FIGURE

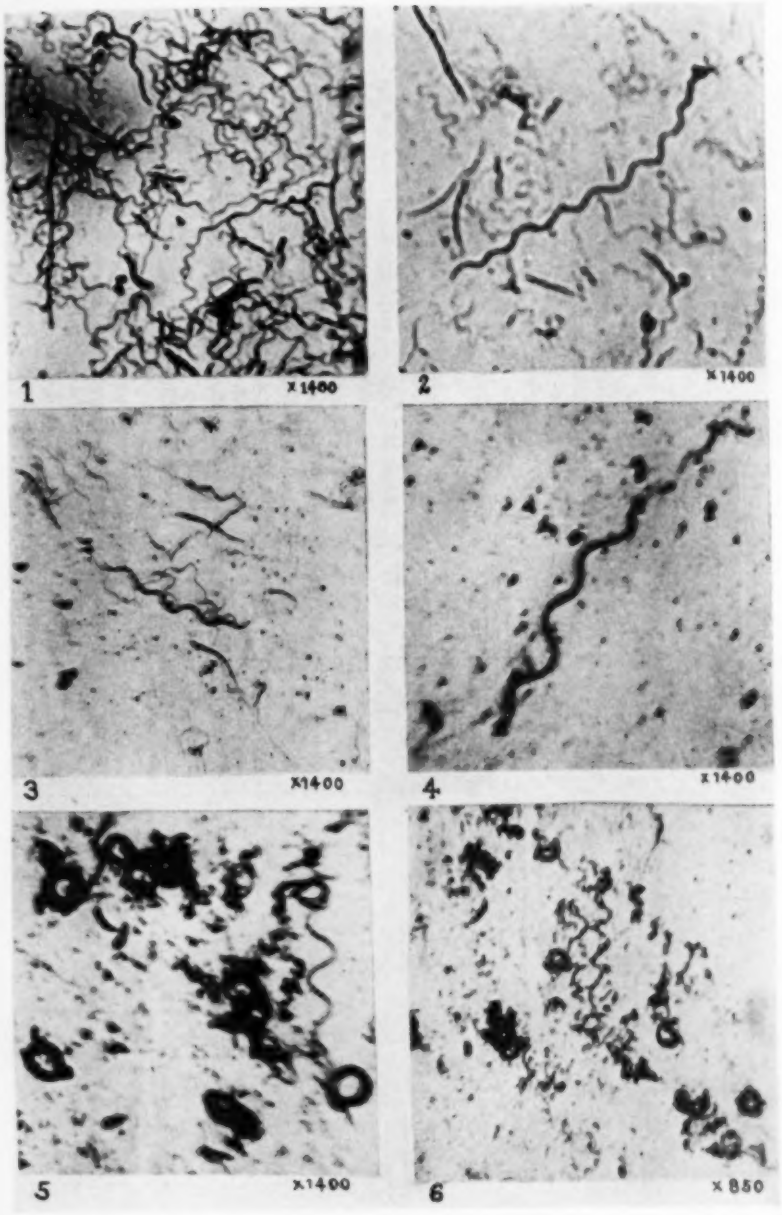
1
x1400
2
x1400
3
x1400
4
x1400
5
x1400
6
x 850

TABLE 1

Findings in intestinal contents of termites

FAMILY	GENUS	SPECIES	SOURCE	FINDINGS IN INTESTINAL CONTENTS
Kalotermitidae	*Cryptotermes*	*brevis*	Porto Rico	Many heavily staining spirochaetes having 3 to 4 spiral turns, uniformly pointed at both ends and averaging 25 by 1 micra
		schwarzi	Florida	Apparently three types of spirochaetes present. One not very numerous, having 4 spiral turns and averaging 40.5 by 1 micra. A larger number of smaller o-ganisms of an average of 25 by 0.5 micra and with 3 spiral turns. A small number of another heavily staining organism seen only in tightly wound circular coils
	Kalotermes	*nigriceps*	British Guinea	Examined in stained preparations only. Apparently two types of spirochaetes present in considerable numbers varying in size and number of spiral turns
		angusticollis	California	Relatively small number of spirochaetes present and these are thin, lightly staining organisms with 2 to 3 spiral curves and averaging 18 by 0.2 micra. Actively motile
	Termopsis	*nevadensis*	Oregon	Two types of spirochaetes only. One is very abundant and has 4 to 12 spiral turns, while the other is much shorter and has only 4 very fine spirals
Rhinotermitidae	*Leucotermes*	*tenuis*	British Guinea	Stained preparation only observed. Many spirochaetes of varying form noted. Some large and taking the stain heavily, others noticeabiy smaller and staining lightly

Rhinotermitidae	*Reticulitermes*	*flavipes*	Maryland	An abundance of large heavy staining organisms, 25 by 0.5 micra in size. The ends are pointed and in dark field preparations occasionally one is seen to be motile
		virginicus	Maryland	Apparently the same organisms as seen in *R. flavipes*, except that the majority are actively motile
		hageni	Maryland	An abundance of spirochaetes indistinguishable from those seen in other species native to Maryland
Termitidae	*Nasutitermes*	*morio*	Porto Rico	A few spirochaetes that stain lightly and average 9 by 0.4 microns. Many organisms with four spiral turns having average dimensions of 19 by 0.5 microns and staining heavily

After considerable experimentation with various stains, the following staining technique was worked out and found to give uniform results:

1. Emulsify the gut contents of the termite in a drop of 0.4 per cent saline and expose to the fumes of osmic acid for thirty seconds.
2. Dry in the air or by very gentle heating.
3. Flood the preparation with a 1:5 solution of 50 per cent carbol fuchsin and 50 per cent aniline gentian violet. Stain sixty seconds.
4. Wash in water, dry and examine.

By this method the background is seen to be a light pink and the organisms much darker as they take the gentian violet dye.

Following the above technique for stained preparations, and by the study of supplemental dark field preparations, ten species of termites, representing three of the four families and six genera were examined, with the results shown in table 1.

From this survey it will be seen that all the species of termites examined are infested with spiral organisms of one or another sort. There does seem to be a difference in the flora, however, as there is considerable variation in the size and staining properties of the spiral organisms observed. It might also seem that there is a difference in the flora when we consider the feature of motility, but we are inclined not to stress this as a differential character, as more extensive observations might indicate motility in all cases. In the cases of those organisms seen to be motile, the motion is that typical of spirochaetes in general, although few of these organisms have been observed to move in any direction other than straight forward or backward. In no stained preparation has there been observed any structure similar to a crista or an undulating membrane and thus far no flagella have been demonstrated.

The accompanying photographs depict typical spiral organisms as observed in various species of termites.

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CLOSTRIDIUM FLABELLIFERUM: A PUTREFACTIVE ANAEROBE WITH BRUSH-LIKE SPORANGIA

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In the summer of 1921 a morphologically peculiar anaerobe was isolated from a table on which hams are handled in a large packing plant in the Midwest. The most striking feature of this organism is the retention by the spore of the vegetative cell, or some part thereof, in the form of a brush-like process made up of innumerable fibrils. This unusual type of sporangium, staining readily by any of the common methods, furnishes a very striking picture especially in older cultures, and contrasts sharply with *C. sporogenes* which rapidly develops free spores. This typical picture has led to such designations as fish tailed and fan tailed spores; hence, the proposed name *Clostridium flabelliferum*. This feature of the organism is constant in favorable media, and in various kinds of media. It is not the result of unusual environment, or unfavorable conditions of growth, but appears to be a normal character of the organism which it has retained for a period of three and one-half years after isolation and culturing in artificial media. A preliminary study has been made of the more important morphological, cultural and biochemical characters and these will now be given as a first step toward a complete description.

MORPHOLOGY

This organism is rod-shaped, with rounded ends, of moderate size, ranging from 3 to 5 by 0.4 to 0.5μ. It forms oval, subter-

minal spores in practically all ordinary media, appearing readily in egg-meat medium within twenty-four to forty-eight hours at 37°C. The sporangium is at first definitely formed, with the edge entire, and well rounded on the distal end (Fig. 1). The spore is larger than the rod, causing one end of the rod to be swollen, with a well-defined tip at the spore end. After two days at 37°C, the sporangium begins to break up into a fan-shaped formation (fig. 1). At first an indefiniteness of outline and a slight flaring of the cell at the end farthest from the spore may be observed. Occasionally the vegetative portion of the sporangium becomes bifurcated, or the longitudinal splitting may give rise to two or more distinct fibrils diverging from the main body of the cell. By the fourth or fifth day, practically all the spores show some degree of this change in sporangium, and the fan-shape is apparent in all degrees of perfection. This condition persists in the egg-meat medium for as long as two months at 37°C. and five months at room temperature, the limits of observation, beyond which time only few free spores, without frayed, fan-like, sporangia are to be found.

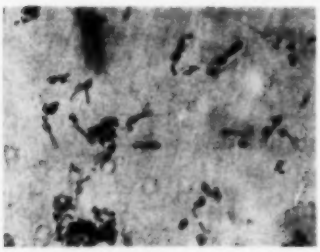

FIG. 1. CLOSTRIDIUM FLABELLIFERUM

Grown in egg-meat medium showing different stages of "fan tail" formation

This peculiar property is in itself distinctive, especially since the character of the spore continues under favorable conditions for a time well past that when the sporangia of other similar anaerobes have become disintegrated and have dropped from the spore, leaving the latter free. It is remarkable, also, that but few so-called free spores have been found in the medium even after two months incubation in meat medium. It seems that the splitting of the sporangium is usually the end of the disintegrative process normally expected in old cultures of spore-bearers. Since this feature is constant, and since it is shown in other culture media, such as plain and glucose broth (four-day cultures), glucose agar (several days old), such fish-tail forms being exactly similar to

those observed in egg-meat medium, this character is deemed of sufficient importance to distinguish the organism from all others. No other anaerobes, or aerobes, have in our experience shown such a peculiarity.

MOTILITY

In ordinary hanging drop preparations, this organism is actively motile in twenty-four-hour egg-meat cultures. The number of flagella has not been determined.

STAINING PROPERTIES

Gram's stain is retained, but is easily decolorized if care is not exercised. When compared with *B. coli* in the same smear, it is decidely Gram-positive when the alcohol is allowed to remain on but a short time. It stains readily with the usual aniline dyes. The frayed sporangium is quite clearly stained when any of the ordinary laboratory stains are used, but shows particularly well with fuchsin. No preliminary treatment with a mordant is required.

COLONIES

Depth colonies. These colonies in one per cent agar shake cultures are wooly and irregular in outline. They resemble *C. sporogenes* colonies. They grow to within about one-half inch of the surface of the agar, the growth in plain and sugar agar being quite luxuriant.

Surface colonies. On the surface of glucose agar (1 per cent glucose, 1.6 per cent agar) growth is obtained within twenty-four to forty-eight hours (in Novy jar by alternate hydrogen and evacuation method) and by the third day present rather coarse, raised colonies with long, intertwining outgrowths. These colonies, too, are similar to those of *C. sporogenes.* In testicular extract agar, the colonies appear tetanus-like, with faint outgrowths, leading to irregular sunbursts in an irregular zone frequently as wide as the diameter of the colony.

CULTURAL CHARACTERS

Egg-meat. In this medium, from which free oxygen was expelled by boiling, growth takes place readily, even when incubated aerobically, within twenty-four hours, with the evolution of slight amounts of gas. Even in this short period of incubation, some few spores are produced, the sporangia of which are fully formed and with edges entire. There is also a very slight odor of putrefaction, which becomes more pronounced on further incubation. The meat shows the usual darkening and breaking up into finer particles such as is shown also by *C. sporogenes* and other putrifiers. This disintegration progresses until half of the medium has been liquefied, when the action is practically stopped. By this time, the meat has become dark brown, with a dirty, amber-colored supernatant liquid.

Milk. Milk, incubated anaerobically under solid paraffin, is readily digested in forty-eight hours. There is at first a soft clot, followed by digestion of the precipitated casein. By the fourth day about half of the medium has been digested, the resulting liquid above the undigested portion being of a dark, amber color. With longer incubation, little or no change takes place.

Gelatin. Plain and glucose gelatin, inoculated and incubated aerobically immediately after autoclaving, are liquefied within twenty-four hours at 37°C. There is no apparent difference between the times when both are liquefied.

Broth. There is rapid and abundant growth in both plain and glucose broth, incubated under anaerobic condition sunder solid paraffin at 37°C. In the latter there is also copious gas production. The growth first appears uniformly distributed throughout the medium but partially settles out after thirty-six to forty-eight hours. The growth becomes abundant and has a tendency to flake out leaving the broth quite clear.

BIOCHEMICAL REACTIONS

Fermentation tests. Preliminary observations on the fermentation of sugars are not entirely conclusive. In the main the

new organism agrees with *C. sporogenes*. Glucose, levulose, galactose, maltose and sucrose are fermented with gas production. Further observations will be necessary before it can be stated whether the two organisms differ in their carbohydrate utilizations.

Glucose-consuming power. In glucose broth containing 0.87 per cent glucose, the sugar was reduced to 0.51 per cent in four days when incubated under anaerobic conditions, at 37°C. *C. sporogenes*, under similar conditions reduced glucose from the same figure to 0.49 per cent in the same time. These figures are so close as not to be significant in separating the two organisms. The quantitative method of Benedict[1] was used.

Peptone-consuming power. The Sorensen test has been shown to be sufficiently accurate to determine relative degrees of the peptolytic power of anaerobes, and was used here to show the peptolytic power of this organism as compared with that of *C. sporogenes*. It has been found, incidentally, that the figures obtained for *C. sporogenes* in this test are surprisingly close to figures reported two years ago by the junior author, under different laboratory conditions and with different reagents, showing that the method is sufficiently accurate for this purpose. Plain peptone-beef extract broth was used. The original medium had a Sorensen figure of 44 (number of cubic centimeters of N/20 NaOH per 100 cc. of medium). After four days growth of the new organism at 37°C., this figure was raised to 116. The difference is 72 according to this scale. *C. sporogenes* gave a figure of 122 when grown under the same conditions: the increase over control is 78. Of the two strains studied therefore, the *C. flabelliferum* had slightly less peptolytic action.

Pathogenicity. This organism is non-pathogenic for the ordinary laboratory animals, namely, mice, guinea pigs and rabbits. A 1:10 suspension of a two weeks old culture in egg-meat was used for the injections, which were made intraperitoneally. No ill effects whatever were noted.

Salt relation. It is of practical interest to note the salt tolerance, with special emphasis on the minimum concentration that

[1] Journal of Bacteriology, 1924, **9**, 13.

will inhibit growth. Tubes of egg-meat medium containing the following concentrations of NaCl were inoculated with the organism and incubated aerobically at 37°C; 1, 3, 4, 6, 8, 12, and 16 per cent. Two series were inoculated, one with *C. sporogenes* and the other with the new organism. After three days incubation, the following conditions were observed:

1. Marked putrefaction, in concentration up to and including 6 per cent NaCl in both series. After twenty days incubation, a slight odor was observed in 8 per cent NaCl. Smears show many vegetative cells in tubes containing as high as 12 per cent salt in spite of the negative odor. Smears from the 16 per cent salt tubes showed occasional vegetative cells. Very few spores were found in any of the salt media, even after two months incubation. This is in agreement with our previous observations that, in general, spore formation either by halophils or other organisms is inhibited by NaCl.

Ham souring experiment. Attempts to produce a "sour ham" by means of *C. flabelliferum* have been negative. A suspension[2] made from an egg-meat culture was injected into one ham and three shoulders about one half hour after the slaughter. The paired ham and shoulder was in each case labelled "control" and was kept, uninoculated, for comparison. All hams and shoulders were hung in a chill room one hour after the inoculation was made and subsequently cured according to the regular packing house routine. Observations were made with a trier after the hams had been in cure for a month and the packing house inspector was unable to detect any sour odor either in the controls or in the inoculated pieces.

This experiment is not considered altogether conclusive. It is known to be rather difficult to produce a typically "sour" ham experimentally. The heaviest inoculation used represented only 0.75 cc. of the original culture and the others considerably

[2] This suspension was made by emulsifying 5 cc. of an old egg-meat culture in 45 cc. of physiological saline, filtering through cotton and centrifuging. The putrid fluid was then poured off and the sediment containing most of the bacterial cells was resuspended in 100 cc. of saline. For injection into the shoulders 3, 7, and 15 cc., quantities were used and 7 cc. for inoculating the ham. Injections were made at the points where experience shows that souring is most apt to occur.

less. The majority of the organisms were in the spore stage and their subsequent development may have been slower than would have been the case with vegetative cells. We are not justified in saying that under certain conditions this organism might not be capable of causing a "sour ham."

SUMMARY

A morphologically distinct species of putrefactive anaerobe has been isolated and studied. It is characterized by the formation of brush-like sporangia and the persistence of these forms even in two-month-old cultures.

It further differs from *C. sporogenes* in the appearance of broth cultures in which the growth rapidly settles out leaving the media clear.

No marked biochemical differences have been noted between the new organism and *C. sporogenes.*

The characters noted above have remained perfectly constant over a period of three years and seem to warrant a new species for which the name *Clostridium flabelliferum* is proposed.

THE MULTIPLICATION OF YEASTS AND YEAST-LIKE FUNGI IN SYNTHETIC NUTRIENT SOLUTIONS

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Numerous papers have been published during the past few years on the propagation of yeasts in inorganic salt-sugar media. Studies in this field are of great import since they may form the bases for a better understanding of the fundamentals of nutrition not only of this interesting group of microorganisms but of the higher organisms as well. The literature on the subject has become rather voluminous. Since it would be impossible to review it in this paper, a review has been prepared for publication in another place. It might be stated, however, that interest in the subject was quickened by the announcement of Wildiers in 1901 that a special substance of unknown chemical composition was necessary for active multiplication of yeasts.

Before presenting the results of our own experimental work, we wish to discuss a few points which seem pertinent after an analysis of some of the reports in this field of research. Such a survey shows that different conceptions of the term growth have been used. These are probably best defined by the methods which have been employed for following the behavior of yeasts in inorganic salt-sugar media. It is quite evident that the methods which have been used have greatly determined the results which have been obtained. A microbiologist sees an evident error in the use of one or two species of yeasts. Several investigators in this field have used but one species in a single medium and generalized to include all of the different species. Some have refuted the data and conclusions from other laboratories secured with a particular species and medium, with data

secured by themselves with another medium and species. Eddy and Stevenson (1920) reported that they investigated some thirty strains of yeast in connection with the development of their yeast test; they did not, however, state whether they used different species or different strains of one or a few species.

It seems necessary, also, to make a sharp distinction between the terms growth, multiplication, propagation, development, proliferation, etc. One must distinguish between data collected by the following methods:

1. Microscopic cell counts
2. Plating in solid media
3. Counting stained smears
4. Weighing increase in yeast mass
5. Measuring the volume of yeast mass
6. Measuring some product of growth or fermentation
7. Nephelometer methods
8. Nitrogen determinations
9. Viability tests on laboratory media

Ide had somewhat the same idea in mind when in reply to a paper by McDonald and McCollum (1921) he pointed out that there were two kinds of proliferation of yeast, one very slow without "bios" and one fast with "bios." Ide claimed that between the two there was such a difference that no one could mistake it once he had seen it.

Some recent work by Henrici (1922) on the bacteria has a significant relation to this discussion. He showed by biometric methods that cells of bacteria grew in size during the so-called lag phase in which it is ordinarily considered that no growth is taking place. In many of the reports in this field of research growth has been followed by means of counting methods. Such methods really depend on cell division or multiplication, functions which may be quite different from growth. It is possible to have growth without cell division. Bachmann (1919) measured carbon dioxide formation as an index of growth. Such a method is accurate if growth and fermentation are always proportional. The work of Henrici, just mentioned, is also sup-

ported by data reported by Clark and Ruehl (1919). They reported striking changes in morphology of bacteria especially during the early stages of growth (from two to six hours). The cells from young cultures of bacteria were generally smaller than those from old cultures. These data indicate that the cell size may increase after multiplication has ceased. While such data on bacteria lend support to this argument, it is fortunate that we have data for yeasts themselves. Slator (1923), after enumerating the various methods that may be used for following the development of yeasts, made the following statement:

> The results obtained by these various methods are sometimes different. For instance, if the growth of yeast in wort is followed, the number by counting stops when the alcohol concentration reaches about 3 per cent, but by centrifuging, growth is shown to continue longer, owing to an increase in the size of the cells. An increase of 20 per cent in amount is often observed after the number has reached a maximum.

A statement from such an authority as Slator indicates the futility of comparing data on "growth" of yeasts which have been secured by different methods. This suggests that some of the confusion and misunderstanding in this field may be due to the lack of a proper understanding of the limitations of the various procedures which have been used.

Then, one may ask whether "growth" is the only criterion of the response of the yeast plant to such a substance as "bios." The term "yeast growth stimulant" might seem to rule out a discussion of this question, but it is apparent that yeast may grow and multiply even though it is not healthy. This fact was mentioned by Wildiers. Wildiers found that yeast would grow slowly in the synthetic media but that the cells were not like normal cells. They were not "healthy." Investigators, especially those in America, have overlooked this factor. German fermentologists have always considered it. A medium which does not permit the development of healthy cells even though it may permit a slow multiplication, is probably not a good one.

We ought also to direct attention to the fact that certain of the vitamin extracts which have been used probably contained food materials of more definite chemical constitution than "bios" is supposed to be, which stimulated growth. It is very difficult to know just how far an extract should be diluted or how it should be prepared perhaps, to contain only "bios" and no other organic matter which would influence growth. The cells of microörganisms are very small. Assume the size of an ordinary yeast cell to be 5μ in diameter. It may be greater or less than this but the above size is not far from an average for *Saccharomyces cerevisiae* which is usually spherical or oval. The amount of solid matter in a cell would be computed as follows:

5μ = 0.005 mm.
Vol. Sphere = $4.1888r^3$.
r^3 = 0.000,000,015,625 cu. mm.
r^3 4.1888 = 0.000,000,065,45 cu. mm. in sphere.
Specific gravity may be assumed to be 1.
0.000,000,065,45 cu. mm. = 0.000,000,065,45 mg.
85 per cent of cell is water. 15 per cent = solid matter.
15 per cent of 0.000,000,065,45 = 0.000,000,009,817,5 mgm. solid matter in a yeast cell 5μ in diameter.

It is easily seen that the vitamin extracts have never been diluted sufficiently to avoid the possible addition of amounts of chemically definite organic matter sufficient to promote growth and multiplication or at least to allow it to start. Once it has started other factors may enter.

EFFECT OF BACTERIAL CONTAMINATION

Not many data are available on the effect of contaminating bacteria on the growth of yeast in the pure synthetic solutions. It is interesting, however, to note that Williams reported better growth of this yeast when the solutions became contaminated with bacteria. However, from the description of his yeast method, which Williams published in 1920 and which is apparently his last contribution to the subject, it is evident that he did not have a pure culture. He weighed 0.300 gram of fresh Fleischmann's yeast (small cake in tin foil) taken from the center of the cake. It is well known to bacteriologists that Fleischmann's

yeast as used by Williams, is contaminated with bacteria. Consequently Williams' data may have been greatly influenced by contaminating bacteria. This influence may either be antibiotic or symbiotic depending upon the type of bacteria which happened to be present. Many of the investigators have not stated specifically just how they protected their media from bacterial contamination or just how they determined that bacteria were not present in their culture flasks. Some of these factors are now being studied in this laboratory.

EXPERIMENTAL

A study of the literature indicates that only a few yeasts have been used in the work on accessory substances in yeast metabolism. Most of the work has been done with *Saccharomyces cerevisiae* from Fleischmann's compressed yeast. In but a few cases have other species been used. Fulmer and Grimes (1923) used *Saccharomyces cerevisiae*, *Torula sphaerica*, and a *Mycoderma*. MacDonald (1922) used five different strains from probably not over two species. A microbiologist sees an evident weakness in generalizing from data secured with but one or a few species of yeasts. However, it is realized that if a standard biological procedure for testing for the presence of accessory substances in materials should be devised, probably a single species of yeast would have to be accepted as the standard test organism.

In this investigation we were interested in the following: First, the use of media containing no "bios" or other accessory substance; and secondly, whether we could secure the growth of a great many different species of yeasts in such media with the technic proposed by other investigators. It must be pointed out that all of our inoculations were large. The question of single cell inoculations is now being studied.

While several different media could have been selected Fulmer and Nelson's Medium F incubated at 37°C. Naegeli's and Fermi's solutions were chosen. Fulmer's Medium F was selected because it seemed to have resulted from a serious attempt, based on considerable experimental work, to find a suitable

medium for yeasts. It did not result from a mixture of chemical substances which were assumed to be suitable. Medium F had the following composition[1] and was handled exactly as by Fulmer and Nelson: Ammonium chloride, 0.188 gram; calcium chloride, 0.100 gram; sucrose, 10.00 grams; dibasic potassium phosphate, 0.10 gram; dextrin, 0.60 gram; distilled water, 100.00 cc. and temperature 37°C. All chemicals were the purest obtainable. The ammonium chloride content of this medium was adjusted for incubation at 37°C. Some of our confirmatory cultures were held at 30°C. This medium when prepared according to the above formula, had a flocculent precipitate of insoluble matter probably calcium phosphate. This was left in the culture flasks since its disappearance served as one criterion of multiplication. When the yeasts multiplied and grew in this medium acids, which dissolved the precipitate, were often formed from the sugars. The medium was placed in clean, sterile Erlenmeyer flasks in amounts of 49 cc. and sterilized in the autoclave. To start the cultures suspensions of the yeast from glucose agar slants were prepared in physiological sodium chloride solution and a small amount added to the first flask. After that the technique of Fulmer and Nelson was strictly adhered to. One cubic centimeter of the culture in the flasks was used for inoculating a sterile flask. Incubation periods of three days at 37.5°C. were used. Frequently plates were made from these flasks in order to insure that the cultures were pure and free from contaminating bacteria and that there was a distinct increase in the numbers of cells. This method, then, was really a measure of multiplication ability and not of growth in the strict sense. Some of the pure cultures grew very slowly in both the test medium and on the glucose agar plates. Culture no. 41, *Torula rubra*, for instance, was a good example. This suggests the necessity of making adequate observations before stating that no growth occurred.

The following pure cultures were used in the preliminary qualitative work. Some of the cultures were lost and were not included in the quantitative studies.

[1] This is the formula for incubation at 30°C.

1. Burgundy wine
2. Champagne yeast
3. *Cryptococcus agregatus*
4. *Cryptococcus glabratus*
5. *Cryptococcus Ludwigii*
6. *Debaromyces tyrocola*
7. *Endomyces albicans*
8. *Endomyces javanensis*
9. *Monilia tropicalis*
10. *Mycoderma rugosa*
11. *Mycoderma vini*
12. *Oidium albicans*
13. Yeast from oysters—Hunter
14. *Schizosaccharomyces Pombe*
15. *Parasaccharomyces Ashfordii*
16. *Parasaccharomyces Thomasii*
17. *Pichia farinosus*
18. *Pichia membranaefaciens*
19. Red yeast (Snow)
20. *Saccharomyces albus*
21. *Saccharomyces anomolus*
22. *Saccharomyces capsulans*
23. *Saccharomyces carlsbergensis*
24. *Saccharomyces cerevisiae*
25. *Saccharomyces ellipsoideus*
26. *Saccharomyces hominis*
27. *Saccharomyces intermedius*
28. *Saccharomyces logos*
29. *Saccharomyces mandshuricus*
30. *Saccharomyces marxianus*
31. *Saccharomyces neoformans*
32. *Saccharomyces Pastorianus*
33. *Saccharomyces* spec—Plimmer
34. *Torula colliculosae*
35. *Torula communis*
36. *Torula cremoris*—Hammer
37. *Torula datilla*
38. *Torula glutinis*
39. *Torula humicola*
40. *Torula mucilaginosa*
41. *Torula rubra*
42. *Torula sphaerica*
43. *Willia anomala*
44. *Willia belgica*
45. *Willia saturnus*
46. *Zygosaccharomyces bisporus*
47. *Zygosaccharomyces chevalieri*
48. *Zygosaccharomyces mandshuricus*
49. *Zygosaccharomyces pastori*
50. *Zygosaccharomyce* Priorianus
51–72. 22 cultures of yeast-like fungi from sore throats (Tanner and Dack, 1924); probably Monilia

Our first object was to determine whether the yeasts would multiply actively in the synthetic media adopted. This was done by inoculating the medium in 50 cc. quantities from a salt suspension of cells grown on agar slants. Growth was determined by the preparation and counting of glucose agar plates, by appearance and by the odor. Some of the species had a tendency to climb the walls of the container after growth had progressed. This made a good additional criterion of growth and multiplication. Several species formed pigments also.

FULMER AND NELSON'S MEDIUM F

The first inoculation into Fulmer and Nelson's medium was made October 1, 1923. Transfers were made every three or four days until March 28, 1924. Twice during this period one week elapsed between subculturing (Thanksgiving and Christ-

mas). When it was decided to include Nägeli's and Fermi's solutions in the work a period of one week between sub-cultures was decided upon. There was a total of about 57 transfers. Early in the fall of 1924, the yeasts were again put in Fulmer and Nelson's medium. These cultures were again started from agar slants. The counts, however, shown in the tables were made on a series of flasks which were being carried along to determine whether these media would support the yeasts. All of them grew although there were differences in the intensities and rates of growth. At frequent intervals the yeasts were counted using glucose agar to determine the multiplication rate. When flasks which seemed to be poor in growth, were left at room temperature, growth was frequently accelerated, showing that 37°C. was not an optimum temperature for all of the cultures used. Although no special experiments were carried out to prove it, it could be seen that after several transfers in Fulmer and Nelson's medium, the cultures grew more quickly as if they were accustomed to it. Finally to secure quantitative data, one set of flasks was chosen for enumeration of the number of cells. These data are shown in table 1.

Examination of the data in this table leads to the conclusion that yeasts are able to multiply in the pure inorganic salt-sugar media of Fulmer and Nelson if the initial inoculation is sufficiently heavy. The multiplication in this medium was markedly superior to that in Nägeli's or Fermi's medium. Eddy and Stevenson also found Fulmer's medium to be better than Nägeli's. The quantitative data shown in the several tables should probably not be taken to show much more than that these budding fungi were able to multiply in the mediums used. The lower counts recorded for some species were probably due to slow growth.

All flasks displayed good multiplication and were grown for a period of about four weeks in the fall of 1924, transfers being made every week except with the last set which was allowed to run for eleven days. As a rule the yeast growth settled to the bottom of the flasks producing a layer of heavy white sediment. On agitation a milky suspension was formed. In some cases the

sediment was more granular or flocculent than in others. Some of the yeasts produced their natural pigment; in two cases the surface of the medium was partially filled with gas bubbles. All of this might be taken as indicating good multiplication. Yeasty odors were produced in all except four cases, namely, 39, 42, 44 and 46.

Another experiment was made to determine the effect of adding accessory substances to medium F. Two sets of flasks prepared as outlined above were sterilized. To one set 0.5 cc. of sterile yeast water prepared according to the instructions of Fred, Peterson and Davenport (1920) was added. Counts were made over a weeks time and the results plotted to determine the slope of the curves. The results of this experiment were not convincing. In some cases there seemed to be a real stimulation on the addition of the yeast water, as if some accelerating substance had been added, while in other cases the slope of the curve was less pronounced and might have been due to the food substances which were present in the yeast water and not so much to an accelerating substance. These curves will not be published at the present time since more work will be done on this question.

Those who have been unable to confirm Fulmer's work have suggested that he used impure ingredients in his medium (Robertson and Davis, 1923). These authors suggested that the cane sugar might be a source of bios. To meet this criticism Fulmer and Nelson extracted some cane sugar for seven days with 95 per cent alcohol in a continuous extractor. Media prepared from this sugar did not give poorer growth nor did the alcoholic extract increase growth when it was added to media. Fulmer and Nelson used 95 per cent alcohol for the extraction. It might be stated that Williaman and Olsen found that 95 per cent alcohol was a poor solvent for bios and that an 80 per cent solution was best. Fulmer, Nelson and White (1923) in a fine piece of work brought very strong experimental data to support their former conclusions that "bios" is not necessary or if necessary may be synthesized by the cell. They prepared methose, a carbon-nitrogen compound of wholly synthetic origin, and found that it could be substituted in place of cane sugar in their

TABLE 1

Showing progress of multiplication in Fulmer and Nelson's Medium F

CULTURE NUMBER	NAME OF YEAST	INITIAL COUNT PER CUBIC CENTIMETER	TWO-DAY COUNT PER CUBIC CENTIMETER	FIVE-DAY COUNT PER CUBIC CENTIMETER	APPEARANCE AT ELEVEN DAYS
1	Burgundy wine	10,000	1,500,000	4,000,000	Heavy growth on bottom
2	Champagne yeast	25,000	120,000	3,500,000	Humpy growth on bottom
3	*Cryptococcus agregatus*	11,000	3,000,000	5,100,000	Heavy growth on bottom
4	*Cryptococcus glabratus*	200,000	2,100,000	2,600,000	Good growth, light, flocculent
5	*Cryptococcus Ludwigii*	600	600,000	2,500,000	Heavy growth on bottom
7	*Endomyces albicans*	300,000	3,000,000	7,700,000	Heavy growth on bottom
8	*Endomyces javanensis*	30	(Slow growth)	5,000,000	Good growth, light, flocculent
9	*Monilia tropicalis*	300,000	15,000,000	2,500,000	Heavy growth, adheres to bottom, milky when agitated
10	*Mycoderma rugosa*	7,600	2,000,000	500,000	Heavy growth on bottom
11	*Mycoderma vini*	200,000	13,500,000	2,500,000	Heavy growth, adheres to bottom, milky when agitated
13	Yeast from oysters—Hunter	3,000	2,300,000	10,000,000	Humpy growth, light, flocculent
15	*Schizosaccharomyces Ashfordii*	800	430,000	3,600,000	Excellent growth, suspended coarse flocs
17	*Pichia farinosus*	200,000	3,200,000	50,000,000	Heavy growth on bottom
18	*Pichia membranaefaciens*	600	700,000	7,500,000	Heavy growth on bottom
19	Red yeast (Snow)	420	2,250,000	5,200,000	Heavy growth, adheres, milky on agitation, reddish color
20	*Saccharomyces albus*	200,000	950,000	400,000	Heavy growth on bottom
21	*Saccharomyces anomolus*	250,000	20,000,000	20,000,000	Heavy growth, bubbles of gas
22	*Saccharomyces capsulans*	800	300,000	100,000	Good growth, light, flocculent
23	*Saccharomyces carlsbergensis*	1,020	(Slow growth)	100,000*	Heavy growth on bottom
24	*Saccharomyces cerevisiae*	960	200,000	500,000	Heavy growth, slight yellow color
25	*Saccharomyces ellipsoideus*	3,500	4,400,000	1,700,000	Lumpy growth on bottom
26	*Saccharomyces hominis*	14,000	500,000	5,000,000	Lumpy growth on bottom
27	*Saccharomyces intermedius*	5,200	4,700,000	1,100,000	Heavy growth on bottom

28	*Saccharomyces logos*	7,000	100,000	1,050*	Coarse flocs covered bottom
29	*Saccharomyces mandshuricus*	12,000	75,000,000	3,500,000	Heavy growth on bottom
30	*Saccharomyces marxianus*	660	1,900,000	9,500,000	Good growth, light, flocculent
31	*Saccharomyces neoformans*	360	1,700,000	100,000	Growth on bottom, one mold, also, was not present when last plate was made
32	*Saccharomyces Pastorianus*	900	6,000,000	1,100,000	Heavy growth on bottom
33	*Saccharomyces* spec—Plimmer	25,000	3,300,000	2,000,000	Good growth, light, flocculent
34	*Torula colliculosae*	22,000	36,000,000	7,300,000	Heavy growth on bottom
35	*Torula communis*	150	(Slow growth)	200,000*	Heavy growth on bottom
36	*Torula cremoris*—Hammar	300,000	72,000,000	1,700,000	Excellent growth, coarse flocs, suspended
37	*Torula datilla*	7,500	300,000	200*	Good growth, light, flocculent
38	*Torula glutinis*	1,800	(Slow growth)	100,000*	Good growth, light, flocculent
39	*Torula humicola*	300,000	15,000,000	1,200,000	Slight growth, surface mold-not present at five-day count
40	*Torula mucilaginosa*	1,850	5,600,000	3,200,000	Heavy growth, reddish color
41	*Torula rubra*	(Too many)	350,000	150,000*	Heavy growth, reddish color
42	*Torula sphaerica*	5,200	165,000,000	100,000	Slight growth, surface mold-not present at five-day count
43	*Willia anomala*	50,000	34,000,000	5,300,000	Heavy growth, bubbles of gas
44	*Willia belgica*	10,000	1,400,000	900,000	Slight growth bottom, mold, not present at five-day count
45	*Willia saturnus*	225,000	20,000,000	19,000,000	Heavy growth, slight red tinge
46	*Zygosaccharomyces bisporus*	7,800	4,500,000	3,100,000	Slight growth bottom, mold, not present at five-day count
47	*Zygosaccharomyces chevalieri*	8,000	8,000,000	900,000	Heavy growth on bottom
48	*Zygosaccharomyces mandshuricus*	200,000	21,000,000	3,000,000	Heavy growth on bottom
49	*Zygosaccharomyces Pastori*	200,000	1,600,000	1,600,000	Heavy growth on bottom
50	*Zygosaccharomyces priorianus*	9,300	5,000,000	900,000	Heavy growth on bottom
51–72	Twenty-two cultures of yeast-like fungi from pathogenic throats gave luxuriant growth in this medium				

* Eleven-day count.

medium. The yeast was able to grow in this solution. Funk and Freedman (1923) also reported a growth-promoting factor for yeasts in cane sugar. While a yeast growth promoting substance may be present in some sugars, one may probably select one which, after study, will be satisfactory for work on yeast multiplication. The sucrose used in these investigations when used in synthetic media would not support the growth of yeast in small inoculations. We know of no other test that may be applied to a substance.

One of the favorable factors for Fulmer and Nelson's medium is probably the insoluble material. This serves as a buffer and removes acids which are formed from the sugar. In many of the synthetic and non-synthetic nutrient solutions developed in former days for the propagation of yeast, a buffer or "neutralizer" was added. It was found that a considerable increase in yield followed the presence of such a compound. As stated above, Fulmer and Nelson's Medium F is the result of considerable study of the effect of the several ingredients. One possible disadvantage, but not a serious one, is the dextrin which causes some inconvenience in preparation.[2] All of the cultures of yeast-like fungi from sore throats gave abundant growth in Medium F.

GROWTH IN FERMI'S MEDIUM

As the investigation progressed it was decided to include several other media. Fermi's medium was introduced first of all. This was prepared as follows: Distilled water, 1000 cc.; magnesium sulfate, 0.2 gram; dibasic potassium phosphate, 1 gram; ammonium hydrogen phosphate, 10 grams; glycerol, 45 grams. It was autoclaved at 15 pounds for ten minutes. The technic of inoculation and observation was identical with that

[2] Fulmer and his colleagues have developed four synthetic media, C, D, E and F. Mediums E and F are especially useful in the cultivation of the budding fungi. Medium E differs from Medium F in the absence of dextrin. Fulmer has advised the authors that Medium E is about as satisfactory as Medium F from the standpoint of yeast crop. Medium E has the advantage that one does not have to bother with the dextrin. Professor Fulmer sent us the following formula for adjusting the ammonium chloride content to the temperature of incubation: 0.188 plus (x°C. − 30°C.) 0.0032.

described for Fulmer and Nelson's medium. In general, multiplication was less abundant in Fermi's medium than in Fulmer and Nelson's. Examination of the data in table 2 indicates an evident difference in ability to utilize the available foods in Fermi's medium. It will be seen that *Saccharomyces carlsbergensis*, *Saccharomyces logos*, *Saccharomyces mandshuricus*, *Torula communis*, *Zygosaccharomyces mandshuricus*, and *Zygosaccharomyces Pastori* did not find Fermi's medium as suitable for multiplication as did certain of the other yeasts. This fact is evidence that one should not select a species of yeast and a medium without first determining the behavior of the yeast in the medium selected. It is also necessary to know the temperature relations of the species in order that cultures may be held at the optimum temperature for growth and multiplication.

In order to determine whether the cells were still viable in the cultures which gave no multiplication in Fermi's medium, 0.5 cc. of yeast water, prepared according to the instructions of Fred, Peterson and Davenport, was added to each of these flasks. It was assumed that the cells might be viable but not able to multiply on account of the lack of the supposed hypothetical bios, or for some other reason. When no multiplication resulted after the addition of yeast water, it could be assumed that there were no viable yeast cells present. In these cases more viable cells were put into the nutrient solution plus yeast water. Several attempts to induce these species to multiply rapidly in the plain synthetic nutrient solution were without results. After the addition of yeast water *Saccharomyces carlsbergensis* and *Saccharomyces cerevisae* showed increased multiplication. When yeast water and more viable cells were added, *Cryptococcus glabratus* and *Saccharomyces intermedius* showed abundant multiplication while the other yeasts showing slow multiplication in table 2 did not undergo a stimulation. The cultures from sore throats gave less vigorous growth in Fermi's medium than in Medium F.

TABLE 2

Showing progress of multiplication in Fermi's medium

CULTURE NUMBER	NAME OF YEAST	INITIAL COUNT PER CUBIC CENTIMETER	TWO-DAY COUNT PER CUBIC CENTIMETER	FIVE-DAY COUNT PER CUBIC CENTIMETER	APPEARANCE AT SEVEN DAYS
1	Burgundy wine	3,000	1,700	3,500	Liquid clear, stringy growth in bottom
2	Champagne yeast	3,000	15,000	3,000	Clear
3	*Cryptococcus agregatus*	4,000	47,000	81,000	Cloudy, light sediment
4	*Cryptococcus glabratus*	300,000	50,000	7,500	Clear
5	*Cryptococcus Ludwigii*	12,000	460,000	194,000	Cloudiness, decided sediment
7	*Endomyces albicans*	5,000	500,000	400,000	Cloudiness, decided sediment
8	*Endomyces javanensis*	1,500	180,000	3,600	Clear liquid, stringy growth in bottom
9	*Monilia tropicalis*	12,000	460,000	400,000	Cloudy with viscid sediment
10	*Mycoderma rugosa*	2,000	110,000	150,000	Cloudy, light sediment
11	*Mycoderma vini*	10,000	150,000	191,000	Cloudy, light sediment
13	Yeast from oysters—Hunter	12,000	510,000	1,120,000	Cloudy, light sediment
15	*Parasaccharomyces Ashfordii*	4,000	80,000	260,000	Cloudy, granular sediment
17	*Pichia farinosus*	1,000,000	3,500,000	7,000,000	Milky liquid, flocculent sediment
18	*Pichia membranaefaciens*	54,000	200,000		
19	Red yeast (Snow)	3,000	250,000	154,000	Cloudy, light sediment
20	*Saccharomyces albus*	2,000	138,000	120,000	Cloudy, light sediment
21	*Saccharomyces anomolus*	32,000	2,400,000	2,350,000	Milky liquid, flocculent sediment
22	*Saccharomyces capsulans*	3,000	500,000	12,000,000	Strong clouding, sediment
23	*Saccharomyces carlsbergensis*	5,000	(Slow growth)	(Slow growth)	Clear
24	*Saccharomyces cerevisiae*	5,000	128	50	Clear
25	*Saccharomyces ellipsoideus*	200,000	48,000,000	17,000,000	Strong clouding, sediment

26	*Saccharomyces hominis*	12,000	1,500,000	2,820,000	Cloudy, light sediment
27	*Saccharomyces intermedius*	6,000	300	75	Clear
28	*Saccharomyces logos*	1,000	(Slow growth)	(Slow growth)	Clear
29	*Saccharomyces mandshuricus*	200,000	(Slow growth)	(Slow growth)	Clear
30	*Saccharomyces marxianus*	250,000	900	200	Clear
31	*Saccharomyces neoformans*	3,000	36,000	100,000	Cloudy, light sediment
32	*Saccharomyces Pastorianus*	7,000	108,000	220,000	Cloudy, light sediment
33	*Saccharomyces spec*—Plummer	2,000	30,000	7,000	Cloudy, light sediment
34	*Torula colliculosae*	150,000	2,000	850	Clear
35	*Torula communis*	900	(Slow growth)	(Slow growth)	Clear
36	*Torula cremoris*—Hammar	250,000	50,000	3,000	Clear
37	*Torula datilla*	10,000	5,000	1,100	Slight sediment, apparently no growth
38	*Torula glutinis*	500,000	50,000	36,000	Slight cloudiness, stringy growth in bottom
39	*Torula humicola*	30,000	2,700,000	530,000	Decided cloudiness, sediment
40	*Torula mucilaginosa*	20,000	262,000	300,000	Cloudy, light sediment
41	*Torula rubra*	1,500	500,000	50,000,000	Strong clouding, sediment
42	*Torula sphaerica*	260	1,100	1,230	Apparently no growth
43	*Willia anomala*	1,000,000	480,000	6,000,000	Milky liquid, large growth
44	*Willia belgica*	78	25,000	725	Apparently no growth
45	*Willia saturnus*	30,000	214,000	468,000	Milky liquid, large growth
46	*Zygosaccharomyces bisporus*	250,000	7,000	600	Clear
47	*Zygosaccharomyces chevalieri*	225,000	8,000,000	24,000,000	Strong clouding, sediment
48	*Zygosaccharomyces mandshuricus*	5,000	850		Clear
49	*Zygosaccharomyces Pastori*	5,000	100		Clear
50	*Zygosaccharomyces priorianus*	5,000	2,000	300	Clear
51–72	Twenty-two cultures of yeast-like fungi from pathogenic throats gave varying amounts of visible growth				

TABLE 3

Showing multiplication in Nägeli's Medium

CULTURE NUMBER	NAME OF YEAST	INITIAL COUNT	THREE-DAY COUNT	FIVE-DAY COUNT	SEVEN-DAY COUNT	APPEARANCE AT SEVEN DAYS
1	Burgundy wine yeast	500			700	
2	Champagne yeast	500			15,500,000	
3	*Cryptococcus agregatus*	1,000			7,200	
4	*Cryptococcus glabratus*	550			Spread	
5	*Cryptococcus Ludwigii*	600			13,000,000	
7	*Endomyces albicans*	800			40,000	
8	*Endomyces javanensis*	200,000	4,800,000	1,500,000	750,000	Cloudy§
9	*Monilia tropicalis*	360			50,000	
10	*Mycoderma rugosa*	700			300,000	
11	*Mycoderma vini*	500			180,000	
13	Yeast from oysters—Hunter	1,000			2,500,000	
15	*Parasaccharomyces Ashfordii*	285			1,800	
17	*Pichia farinosus*				240,000	
18	*Pichia membranaefaciens*	200			11,500	
19	Red yeast (Snow)	2,000			400,000	
20	*Saccharomyces albus*	1,000			1,100	
21	*Saccharomyces anomolus*	300			400,000	
22	*Saccharomyces capsulans*	20,000	1,000*	19,000†	500,000‡	Very slight clouding
23	*Saccharomyces carlsbergensis*	30,000	2,000*	5,000†	500,000‡	Cloudy—moderate
24	*Saccharomyces cerevisiae*	150,000	4,500,000	910,000	800,000	Cloudy—with sediment§
25	*Saccharomyces ellipsoideus*	500			6,600	
26	*Saccharomyces hominis*	500			10,000	
27	*Saccharomyces intermedius*	36,000	1,200*	6,000†	1,500‡	Clear
28	*Saccharomyces logos*	200,000	2,000,000	5,000,000	10,000,000	Cloudy§
29	*Saccharomyces mandshuricus*	7,200	2,000*	1,100†	750	
30	*Saccharomyces marxianus*	75,000	8,600*	25,000†	100,000	Very slight clouding

31	*Saccharomyces neoformans*	75,000	12,000*	24,000†	300,000‡	Very slight clouding
32	*Saccharomyces Pastorianus*	500		5,000		
33	*Saccharomyces spec*—Plummer	70,000	11,000*	200,000†	32,000‡	Very slight clouding
34	*Torula colliculosae*	500			12,000	
35	*Torula communis*	200,000	4,500,000	1,000,000	15,000,000	Cloudy
36	*Torula cremoris*—Hammar	500			2,000	
37	*Torula datilla*	15			0	
38	*Torula glutinis*	2,000+			45,000	
39	*Torula humicola*	1,000			150,000	
40	*Torula mucilaginosa*	500			10,000	
41	*Torula rubra*	6,000	2,000*	125†	40‡	Clear
42	*Torula sphaerica*	400			400	
43	*Willia anomala*	1,500			110,000	
44	*Willia belgica*	200,000	1,860,000	3,000,000	1,500,000	Cloudy§
45	*Willia saturnus*	2			5,000	
46	*Zygosaccharomyces bisporus*	1,500			1,000	
47	*Zygosaccharomyces chevalieri*	2,000+			4,300,000	
48	*Zygosaccharomyces mandshuricus*	50			200	
49	*Zygosaccharomyces Pastori*	0			700	
50	*Zygosaccharomyces priorianus*	25,000	8,400*	18,000	16,000‡	Very slight clouding
51–72	Twenty two cultures of yeast-like organisms from sore throats gave very scant growth in Nägeli's Medium					

* Two days.
† Four days.
‡ Six days.
§ Ten days.

GROWTH IN NAGELI'S MEDIUM

In 1879 Nägeli reported three media for studying the nutrition of certain of the lower fungi. That used in this investigation had the following composition: Water, 1000 grams; ammonium tartrate, 10 grams; dipotassium phosphate, 1 gram; magnesium sulfate, 0.2 gram; calcium chloride, 0.12 gram. This medium was sterilized in the autoclave at 15 pounds for ten minutes.

The technic followed with Nägeli's medium was identical with that described above for Fulmer and Nelson's medium. All chemicals were the purest obtainable and every precaution was exercised to prevent the entrance of any extraneous organic matter into the flasks. Counts were not made as frequently as with the two former media.

Nägeli's medium proved to be less satisfactory than either Fulmer and Nelson's Medium F or Fermi's. As would be expected some of the yeasts gave practically no multiplication while others gave excellent indication that the medium was suitable for multiplication.

In table 3 showing the multiplication rate in Nägeli's medium, a number of species gave very slow multiplication. In case of the latter as was done with similar cases in Fermi's medium, 0.5 cc. of yeast water and more viable cells were added to the culture flasks. When this was done *Endomyces javenensis*, *Torula communis*, *Willia belgica* and *Zygosaccharomyces priorianus* showed more active multiplication. The cultures of yeast-like organisms from sore throats also gave very scant growth. The flasks remained either clear or showed only the slightest amount of turbidity.

CONCLUSIONS

Various methods have been used for estimating the multiplication and growth of yeasts in synthetic nutrient solutions. The data from these several methods may not be comparable; this may explain some of the controversies which have arisen in this field of investigation. Distinction should be made between methods which measure growth, multiplication, etc. Yeast cells may grow after multiplication has stopped.

The following conclusions are drawn from the data presented in this paper.

1. Fifty pure species of yeasts and twenty-two strains of yeast-like fungi from sore throats were found to multiply abundantly in Fulmer and Nelson's Medium F over a period of eleven months when cultured according to the technic used by these authors.

2. Fulmer and Nelson's Medium F allowed more vigorous multiplication than either of the other two media employed.

3. Multiplication in Fermi's medium was less abundant than in Fulmer and Nelson's Medium F but perhaps a little more abundant than in Nägeli's medium.

4. Nägeli's medium was the least satisfactory of the three media used.

5. Yeasts and yeast-like fungi seem to be able to develop in pure synthetic nutrient solutions if sufficiently heavy inoculations are used. They may develop more rapidly, however, if substances containing "bios" or organic matter are added.

6. Yeast growth and crop are dependent upon a number of factors such as temperature relations of the yeast, acclimatization to a certain medium, purity of the ingredients, chemical constitution of the medium, aeration, hydrogen ion concentration, etc.

7. Each species of yeast probably has its own dietary requirements which are more satisfactorily supplied by one medium than by another.

8. It is unnecessary to assume the requirement of "bios" or any other hypothetical substance to explain the absence of multiplication of a yeast in a synthetic nutrient solution. Lack of multiplication may be due to other things; conversely, the stimulation of growth and multiplication following the addition of a supposedly "bios" containing substance does not indicate that "bios" is necessary since the stimulation might be due to other factors in the "bios" preparate.

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MORPHOLOGICAL AND PHYSIOLOGICAL VARIATIONS IN THE DESCENDANTS OF A SINGLE DIPHTHERIA BACILLUS

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The question of variation in morphology, and in toxin production among the members of the diphtheria group has been of lively interest to the bacteriologist since Roux and Yersin in 1890 made the statement that "they believed the diphtheria group was but a single species" and declared that the morphological and biological differences between the types, (*Bacillus diphtheriae*, Klebs-Loeffler, *B. pseudo-diphthericus*, Hoffman-Wellenhof, and *B. xerosis*, Neisser), were not sufficient to warrant establishing new species.

In reviewing the literature, a striking difference of opinion in regard to the subject of variation on the part of the different authors was very evident.

We find, however, that practically all authors in reporting their experiments remark that they have made use of a *pure* culture, obtained by the isolation of single colonies, by one means or another. In but one instance has it been reported that a single organism was isolated, hence, many of the conclusions given must have been based on the inference that the colonies studied were the progeny of a single organism. Ørskov (1922) said, and we believe correctly, "that by a pure culture we understand, as is well known, a culture consisting of individuals of which we know with certainty that all are descended from one single cell, and one only."

While it is true that all our methods of pure culture study are based on the idea that colonies are developed, each from a single organism, yet in view of the fact that we cannot always

be sure of this, we have felt that any experiments on variation should begin with the isolation of a single cell. Therefore, in our experiments here reported, we have begun with a pure strain of the diphtheria bacillus, isolated from a single cell, and have traced the morphology of the descendants of this single organism and their virulence, and have further confirmed the work by the isolation of other single cells all descendants of the parent strain.

The isolation of a single organism of any species of bacteria is a question of method. The method of isolation should allow of no criticism as to the purity of the strain obtained. Also, in a pleomorphic group such as the diphtheria group it is necessary to know exactly the type of organism isolated. In a series of experiments using methylene-blue as a vital stain good results were obtained in differentiating the various types of the diphtheria bacillus.

The method of isolating a single organism as used by Ørskov, (1922) was found to be suitable to the problem. This method combined with the use of a vital stain was adopted by the writer in obtaining the pure lines used in these experiments.

All morphological types reported were from preparations grown on Loeffler's blood serum slants having a hydrogen ion concentration of 7.4, unless otherwise stated.

A highly virulent culture of the diphtheria bacillus, used for the demonstration of toxin production, was used as a stock culture from which a single organism was obtained by the Ørskov method.

This single organism was an A_1 type of Wesbrook's classification and will be designated hereafter as the parent organism, while all the descendants of this organism will be known as the parent strain.

Observations made on the morphological types of the first six transplants of the parent strain showed, coccoid forms, or the following Wesbrook types: C_2, D_2, A_1, A_2, B_1, C_1. Some forty subsequent transplants of this parent strain showed the types reported above at some time.

All virulence tests made by the subcutaneous inoculation of

a broth culture of the parent strain into 250-gram guinea pigs were positive, with a typical postmortem picture.

Two other single diphtheria cells, both of A_1, were isolated at the same time from the sixth transplant of the parent strain by the Ørskov method. The descendants of one of these organisms will be designated as daughter strain number one, and the descendants of the other organism will be known as daughter strain number two.

The Wesbrook types that developed on the first transplant of daughter strain number one were C_2, D_2, and B_2, while sixty subsequent transplants showed the A_1, B_1, C_1, D_1, C_2 types. Virulence tests carried on from cultures of this series were all positive and comparable to the virulence exhibited by the parent strain and stock culture.

The Wesbrook types as developed in daughter strain number two were in marked contrast to those seen in the parent strain or daughter strain number one. The first transplant showed the D_2, E_2 types while eleven subsequent cultures showed only E_2 types. Ten other transplants showed again the D_2, E_2 types and the remaining thirty-eight cultures gave the CC_2, DD_2, EE_2 of Wesbrook's types.

The virulence of daughter strain number two was as different when compared to the virulence of daughter strain number one and of the parent strain as had been the difference in the morphological types found. In the case of daughter strain number two all virulence tests were negative. The virulence tests were then repeated using increased amounts of culture up to four cubic centimeters with negative results. Then, to be sure that there was no toxin formed, increased amounts of culture of different ages, from one day to two weeks, were used for inoculation with negative results. Finally, inoculations were made of a culture of daughter strain number two which showed the A_1, CC_2, DD_2, EE_2 types of Wesbrook (obtained from experiments reported later in this paper) still with negative results.

In view of the fact that a number of writers have been able to increase the virulence of a slightly toxic diphtheria strain by the addition of amino acids or enzyme preparations it was

thought that under favorable conditions a toxin might be produced from daughter strain number two by some similar method.

Davis and Ferry (1919) were able to increase the potency of the toxin of a diphtheria strain by the addition of cystine to the medium.

Several inoculations into guinea pigs were made with cultures of daughter strain no. 2. The cultures used in the inoculations were of different ages, twenty-four hours to seven days, and grown in media which contained amounts of cystine varying from 0.005 grams to 0.05 grams per cubic centimeter of medium. Cystine however did not aid in the production of a toxin and the results of the inoculations were again negative.

Similar inoculations were made using cultures grown in media containing killed bacterial emulsions, Kent (1922), and water soluble extracts of commercial yeast, potato, and carrot, Kligler, (1919), Kent (1923), these also gave negative results.

An attempt was then made to enhance the virulence of daughter strain number two by contact with the peritoneal fluid of a guinea pig *in vivo*.

Two cubic centimeters of a twenty-four hour broth culture of daughter strain number two were inoculated into the peritoneal cavity of a three hundred gram guinea pig. At the end of twenty-four hours the peritoneal fluid was withdrawn aseptically and transplanted to Loeffler's serum slants. Examination of a preparation made from this culture and stained with Loeffler's methylene-blue showed a pure culture of small coccoid forms which on the subsequent transplant developed a pure culture of C_1 of the Wesbrook types. This culture was designated as P_1.

Four cubic centimeters of a twenty-four hour culture of strain P_1 were inoculated as above and the peritoneal fluid withdrawn as before. Examination of the first transplant showed small coccoid forms with a few of the Wesbrook types. Subsequent transplants showed A_1, CC_1, DD_2, E and E_2 types of Wesbrook. This strain is designated as P_2. A direct smear of the peritoneal fluid showed A_1, C_2, D_2 types of Wesbrook.

Virulence tests, made by subcutaneous inoculation of broth cultures of strains P_1 and P_2 into 250-gram guinea pigs, were negative.

The body fluid as found in the peritoneal cavity of a normal guinea pig had evidently exerted some action upon the diphtheria organism injected, in that the diphtheria organisms had been changed from the granular and barred forms to small coccoid forms through contact with the peritoneal fluid.

From the above experiments it appears that daughter strain number two has failed to produce a toxin but morphologically has produced some of the types found in the parent strain.

Six other single daughter organisms were isolated from the parent strain by the before mentioned method. These daughter strains were subcultured enough times to confirm the previous experiments, in showing that the morphological characteristics of the diphtheria organism were subject to variation. All of the cultures were virulent.

DISCUSSION

The recognition of the Hofmann, or pseudodiphtheria bacillus, as a distinct species from the diphtheria bacillus becomes more, difficult as bacteriological research advances. Until, however overwhelming evidence is presented to differentiate between these two organisms, there will be those who believe there is but one species, of which the Hofmann bacillus is but a variation or mutation.

Lessieur (1901) and Haven (1920) have shown that the morphological types of the diphtheria bacillus are apparently nonspecific. The latter writer has determined two groups of diphtheria by the agglutination test in which there is no evidence of cross-agglutination, and in both groups has found virulent and avirulent diphtheria bacillus. Wesbrook, Wilson, and McDaniel (1899) suggested, and Gorham (1901) noticed, that the barred types were changing to the solid staining types under the influence of body fluids, and believed the Hofmann bacillus to be a morphological variety of the diphtheria bacillus.

Salter (1899), Ohlmacher (1902), and others succeeded in changing the pseudodiphtheria organism into a typical diphtheria bacillus at the same time increasing its virulence by passage through animals.

Goodman (1908) and Meader (1919) were unable to correlate fermentative power and virulence. The former writer obtained a strain of the diphtheria bacillus which produced no acid in broth.

Williams (1902), Berry, and Banzhof (1912), and others have claimed the non-variability of the diphtheria group.

Rickards (1908) has reported variations in virulence among different colonies of the same strain, which work has been confirmed by Baerthlein, Jacobstal, Bernhardt, and Paneth (1913).

Mellon (1917), in his classification of the diphtheroids has considered the Hofmann bacillus as a mutant of the diphtheria bacillus, and concluded by saying "any of the sub-groups may become pathogenic under favorable conditions."

Dixon (1919) says "transmutation differs from variation in degree alone; it is a question of the extent of the modification and the degree of permanence it exhibits."

Walter (1915) in his chapter on mutation has concluded that "to the student of heredity, there are two distinctions of prime importance with respect to mutations. First, that they usually appear full-fledged without preparatory stages, and second, they breed true from the start. Fluctuations on the contrary ordinarily 'revert' to the parental type in subsequent generations."

Morgan (1919) has shown that the "genes," which are the character determiners, stand at definite levels in the chromosomes.

Morgan, Sturtevant, Muller and Bridges (1922) have said that the phenomena known as "non-disjunction" may be the factor which causes variation. It has been shown that when certain genes were absent from the chromosomes, the hereditary characters controlled by those genes were also absent.

"The loss of any character would necessitate the chemical rearrangement of the internal structure of the organism or the intracellular stereochemic recombinations necessary for

the birth of new forms" (Mellon, 1921). The new forms in the case of the diphtheria bacillus would be the non-toxic organisms.

The experiments of Middleton (1915) on *Stylonchia;* Jennings (1916) on *Difflugia;* and Hegner (1918) on *Arcella dentata* have shown mutations to occur in unicellular organisms. The mutations were here due to an unequal division of the nuclear material and environmental agents. It is probable that the same phenomenon has taken place in the development of the non-toxic daughter strain no. 11 of the diphtheria bacillus.

The preceding experiments have shown that there is a distinct morphological fluctuation in the diphtheria bacillus. What is most important, however, a distinctly non-virulent strain of the diphtheria bacillus was obtained by the isolation of a single organism from a virulent parent strain, also descended from a single organism.

A fluctuating variation has been shown by the development of barred Wesbrook types from the solid forms, but a real mutation has been shown by the development of a non-toxic strain.

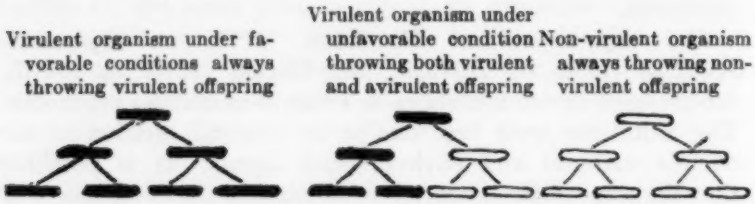
To explain the mutation from the toxic to the non-toxic diphtheria bacillus, we assume that there is present in the bacillus, organized or unorganized chromatin or nuclear material, which as "chromosomes" divides regularly with the division of the organism and imparts to each daughter bacillus the "hereditary character" of producing toxin.

Therefore, it would follow that as long as all factors were favorable to the regular division of the "chromosomes" and "genes" of the diphtheria bacillus, the bacillus would be toxic.

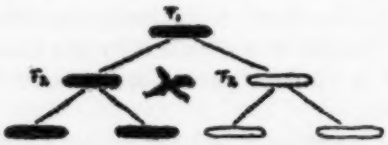
If, however, the factors should become unfavorable to the organism, the process of "non-disjunction" would take place and there would follow an unequal division of the "chromosomes" or nuclear material, and part of the progeny of the toxic diphtheria bacilli would be non-toxic. The "character" of producing toxin would be permanently lost and the non-toxic strain resulting would be permanently non-toxic.

With this in mind, the following diagram has been prepared

to indicate the possibility of mutation from the virulent to the avirulent organism:


Virulent organism under favorable conditions always throwing virulent offspring
Virulent organism under unfavorable condition throwing both virulent and avirulent offspring
Non-virulent organism always throwing non-virulent offspring

In my own experiments the following diagram would illustrate the actual condition.



F_1 would be comparable to the toxic parent strain, and F_2 black and F_2 white would be comparable to the toxic and non-toxic daughter strains nos. 1 and 2 respectively.

Daughter strain 2 would therefore always be non-toxic, and any increase in virulence of a non-toxic diphtheria bacillus or pseudodiphtheria bacillus, as has been reported must have been according to our experiments, due to a mixed culture.

We feel that we are justified in saying that the non-virulent diphtheria bacillus is a direct mutation of the toxic diphtheria bacillus.

From the above observation we have concluded that:

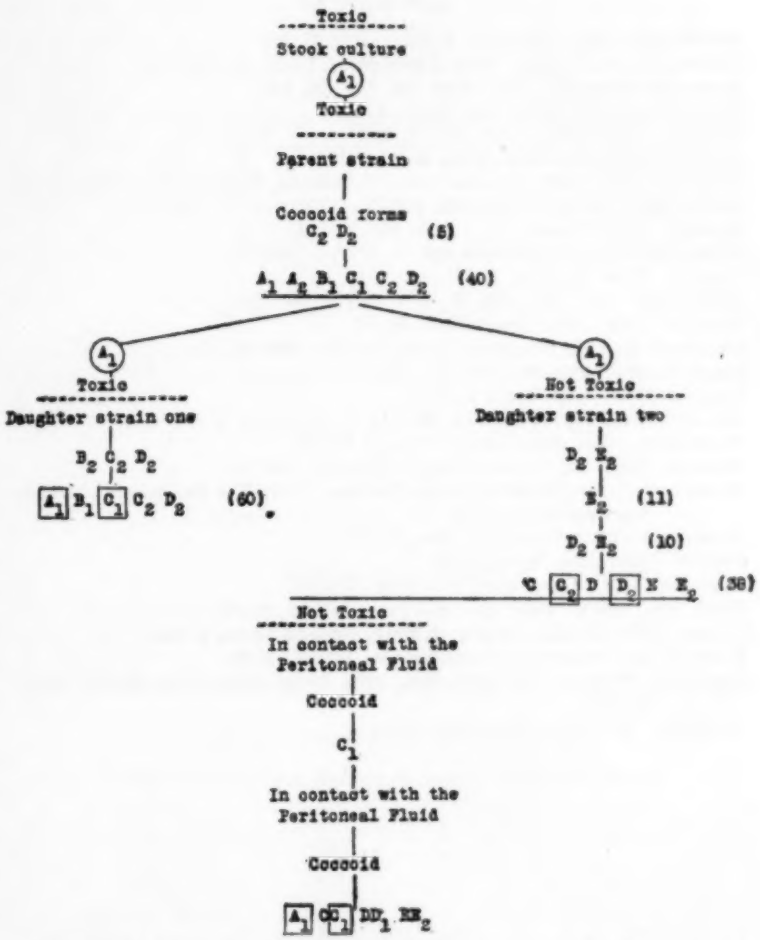
Morphological types of the diphtheria bacillus have no hereditary significance and have no relation to virulence.

The descendants of toxic diphtheria organism may be either toxic or non-toxic.

The non-toxic strains are direct mutations from toxic strains.

The non-toxic strains are permanently non-toxic.

The above conclusions are based on the study of pure line strains obtained by the isolation of a single organism.



The types of organisms and the toxicity of the strains has been indicated in the diagram above. The single organisms isolated are noted by a circle, the numbers indicate the number of transplants made.

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